UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

BIOQUÍMICA

PERFIL DA ATIVAÇÃO DOS ASTRÓCITOS EM DIFERENTES MODELOS BIOLÓGICOS

Paula Santana Lunardi

Porto Alegre

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Paula Santana Lunardi

Orientador: Carlos Alberto Gonçalves

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial para obtenção do título de doutor em Ciências Biológicas: Bioquímica

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“What is the function of these glial cells in neural centers? The answer is still not known and the problem is even more serious because it might remain unsolved for many years to come until physiologists find direct methods to attack it.”

Santiago Ramón y Cajal

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Apresentação

Os resultados desta tese estão apresentados sob a forma de artigos científicos, em dois capítulos (Parte II). As seções Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios artigos.

Os itens Introdução (Parte I), Discussão e Conclusão (Parte III), encontrados nesta tese, apresentam interpretação e comentários gerais sobre os capítulos contidos neste trabalho. As referências contidas no final da tese referem-se somente às citações que aparecem nos itens Introdução e Discussão.

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PARTE I

Resumo

Apesar de representarem cerca de 50% de todas as células do encéfalo humano, as células da glia foram negligenciadas pela Doutrina do Neurônio, no início do século XX, por não serem excitáveis e por não se comunicarem como os neurônios. Durante um longo período, os estudos sobre os astrócitos se restringiam a suas funções de suporte do SNC. Atualmente se sabe que os astrócitos expressam receptores para uma variedade de neurotransmissores, como por exemplo, receptores colinérgicos nicotínicos, sugerindo possíveis mecanismos de resposta aos sinais enviados pela atividade neuronal, de modo variável e dinâmico, espontaneamente ou dependente da atividade neuronal e ativado pela alteração dos níveis intracelulares de Ca^{2+}. Entretanto, os mecanismos específicos que explicam como essas células podem ser ativadas e qual sua contribuição para funcionamento do SNC ainda não estão totalmente esclarecidos. A secreção da proteína trófica S100B pelo astrócito pode ser um exemplo de comunicação celular, bem como também marcador biológico para diversas doenças. O nosso grupo já mostrou o envolvimento de diversos sistemas neuronais na modulação da secreção da proteína S100B. Um outro exemplo de comunicação é a liberação de gliotransmissores e a influência aguda astrocítica na transmissão sináptica. O objetivo dessa tese foi investigar se ativação do sistema colinérgico modula a secreção da proteína S100B em culturas primárias de astrócitos. Ainda, investigamos o uso da optogenética para o estudo da gliotransmissão em fatias de hipocampo. As culturas foram tratadas com inibidores da acetilcolinesterase (huperzina-A e tacrina), agonistas (acetiltiocolina, nicotina e carbacol) e antagonistas (mecamilamina e escopolamina) colinérgicos incubados durante 1 e 24 h. Os estudos em optogenética partiram primeiramente da caracterização do sistema cre/lox através da análise da expressão das proteínas de interesse, seletiva para astrócitos, por imunohistoquímica. Os resultados do primeiro estudo mostraram que a huperzina-A aumentou a secreção de S100B em culturas primárias de astrócitos, assim como o tratamento com nicotina; no segundo, discutiu-se as limitações metodológicas quanto a especificidade das proteínas e alteração da fisiologia dos astrócitos. Os resultados obtidos nessa tese ressaltam para a importância do conhecimento da fisiologia do astrócito, contribuindo para o entendimento de um possível mecanismo de ativação colinérgico dos astrócitos em promover secreção de S100B. Também é preciso ressaltar a relevância de novas metodologias que ajudam a descrever melhor o papel dos astrócitos na atividade sináptica.

Abstract

Although about half of the brain cells are glial cells, the Neuron Doctrine has neglected them, at the beginning of 20th century, because they were not excitable and also, they were unable to communicate as neurons.

For a long time, the studies about astrocytes functions were limited to trophic and metabolic support to neurons by which providing for the homeostasis of the nervous system. A variety of studies have been shown the expression of different neurotransmitters receptors in astrocytes, for instance, nicotinic receptors, suggesting the possibility of astrocytic response to neuronal activity. This response is variable and dynamic and mostly can be activated by changes at the intracellular calcium levels. However, the specific mechanisms of astrocytic Ca^{2+} excitability and how this process could contribute to CNS functioning are still unclear. The S100B secretion could be an example of cellular communication, as well as biomarker for diverse diseases. S100B is calcium binding protein, produced and secreted mainly by astrocytes. Our group has already demonstrated the relevance of various neuronal systems mediating the S100B secretion. Another astrocytic form of communication could be the release of gliotransmitters and the acute influence at synaptic transmission. The aim of this thesis was to investigate whether cholinergic system activation could modulate S100B secretion in primary astrocytes cultures. Moreover, we investigated the optogenetic properties as a tool for gliotransmission studies in hippocampal slices. The astrocytes cultures were treated with acetylcolinesterase inhibitors (huperzine-A and tacrine), agonists (acetylthiocholine, nicotine and carbachol) and antagonists (mecamylamine and scopolamine) for 1 and 24h. The optogenetic studies were conducted, firstly, from the cre/lox characterization of protein expression, selectively for astrocytes, by immunohistochemistry. The first study results showed that huperzine-A increased S100B secretion, as well as nicotine; in the second study, we have discussed the main methodological limitations concerning the protein specificity and astrocytes physiology changes. These results raise the importance of astrocytes functions investigations, especially S100B secretion, contributing for the first time to the understanding of astrocytes excitability likely trough nicotinic system activation. In addition, it is worthy to note that new methodologies are relevant and can help us in a better description of astrocytes role during synaptic activity and brain functioning.

Lista de abreviaturas

GFAP	proteína ácida fibrilar glial
NGF	fator de crescimento neuronal
BDNF	fator neurotrófico derivado do encéfalo
NT-3	neurotrofina-3
IP3	inositol trifosfato
ATP	adenosina trifosfato
NO	óxido nítrico
RAGE	receptor para produtos finais avançados de glicação
DMSO	dimetilsulfóxido
ACh	acetilcolina
ChAt	colina acetiltransferase
AChE	acetilcolinesterase
DA	Doença de Alzheimer
IAChE	anticolinesterásicos
HupA	Huperzina-A
nAChRs	receptores colinérgicos nicotínicos
TNFα	tumor de necrose tumoral alfa
VRAC	canais iônicos regulados por volume
SICs	correntes lentas de entrada
SOCs	correntes lentas de saída
LTD	depressão de longa duração
ChR2	canal-rodopsina 2
LiGluR	receptor glutamatérgico ativado por luz
CatCh	canal-rodopsina translocador de cálcio

$CreER^{T2}$	enzima cre-recombinase dependente de ligante
AAV	adenovírus associado
NeuN	antígeno nuclear neuronal
GFP	proteína de fluorescência verde
YGP	proteína de fluorescência amarela
GLAST	glutamato-aspartato transportador

1. Introdução

1.1. Brevíssima história dos estudos sobre os astrócitos

Quando a neuroglia foi descoberta pela primeira vez, havia um debate importante que argumentava se a neuroglia era um tecido de conexão ou uma verdadeira população de células (Somjen, 1988). O termo “glia” foi cunhado na metade do século XIX pelo biólogo alemão Rudolf Virchow para “nomear o material biológico observado ao redor dos neurônios, que parecia proporcionar união e forma ao conjunto” (Virchow, 1859). Este conceito original, que definia a glia como um tecido conectivo, deu origem ao seu conhecido papel de fornecer suporte estrutural ao sistema nervoso central (SNC). De fato, as primeiras imagens detalhadas das células gliais foram produzidas por Golgi, entre 1870 e 1871, através da técnica de marcação em preto ou “reazione nera”. Golgi foi também o primeiro a descrever a existência de contatos entre os processos astrogliais e os vasos sanguíneos do encéfalo (Golgi, 1903). Uma categoria de células neurogliais previamente observadas por Golgi foi denominada “astrócitos” por (Lenhossék, 1891) e foi previamente classificada por (Kölliker, 1902) e (Andriezen, 1893) em astrócitos protoplasmáticos localizados na substância cinzenta e astrócitos fibrosos, na substância branca. Mais tarde, a diversidade morfológica das células gliais foi documentada por Retzius (1894-1916) e Santiago Rámon y Cajal (Figura 1). As ilustrações de Ramón y Cajal, ainda em 1888, mostraram que a astroglia era representada por fascinantes *células aracneiformes* (células com forma de aranha), que ocupavam a maior parte do tecido nervoso de um encéfalo humano. Mais tarde, em 1913, Cajal observou que alguns astrócitos estavam em contato com neurônios, e outros com vasos sanguíneos. Neste trabalho, Cajal

utilizou uma técnica de marcação com ouro (Ramón y Cajal, 1913), o qual se associa a filamentos intermediários constituídos principalmente de proteína ácida fibrilar glial (GFAP). A marcação para esta proteína é reproduzida até hoje, como principal marcador astrocítico (Kimelberg, 2004). É válido notar que apesar dos estudos iniciais sobre os astrócitos, os neurônios tinham sido descobertos apenas há poucas décadas e cientistas do mundo todo estavam tentando decifrar como eles funcionavam. Apesar dos estudos sobre os astrócitos seguirem paralelamente (Garcia-Segura, 2002), Ramón y Cajal iniciou uma série de investigações para entender como os neurônios se comunicavam e foi assim que surgiu sua teoria conhecida como a Doutrina do Neurônio (BULLOCK, 1959; Guillery, 2007). A visão dos cientistas sobre as propriedades passivas dos astrócitos estagnou diante desta doutrina, principalmente devido às diferenças nos mecanismos de excitação destas células quando comparadas aos neurônios. De qualquer modo, nos últimos anos, os exemplos de suas funções específicas e relevantes nos processos fisiológicos normais começaram a se acumular e o número de doenças conhecidas nas quais envolvem a disfunção dos astrócitos está aumentando.

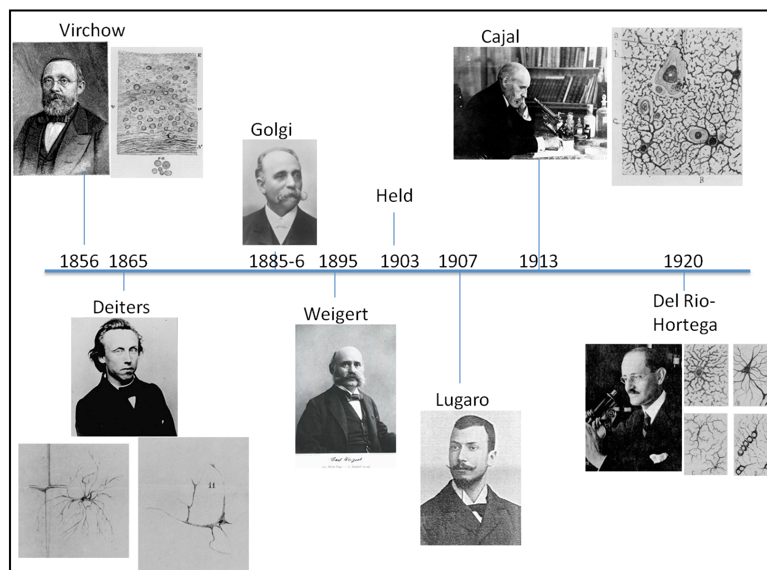

Figura 1. Linha do tempo que resume os principais neurocientistas que participaram da história da glia.

1.2. Classificação e funções gerais dos astrócitos

Astrócitos são as células da glia que ocupam cerca de 50% da substância cinzenta no córtex humano e podem ser tão heterogêneos quantos os neurônios (Koob, 2009). Dentre as diferentes classes, duas principais já estão bem descritas e diferem entre si pela morfologia, fenótipo, localização e por suas funções principais: astrócitos protoplasmáticos, encontrados na substância cinzenta e seus processos envolvem tanto sinapses quanto vasos sanguíneos, enquanto os classificados como fibrosos (fibrilares) da substância branca estão em contato com nódulos de Ranvier e vasos sanguíneos (Iadecola & Nedergaard, 2007; Rossi *et al.*, 2007; Oberheim *et al.*, 2012). Astrócitos estão organizados de modo que seus domínios não se sobrepõem e apenas suas projeções membranosas muito finas fazem contato com a vizinhança (Bushong *et al.*, 2002; Halassa *et al.*, 2007b). Por exemplo, na área CA1 do hipocampo os processos astrocíticos são capazes de alcançar cerca de 40%-60% das sinapses (Witcher *et al.*, 2007; Bernardinelli *et al.*, 2014), e este processo de cobertura das sinapses parece ser regulado pela atividade sináptica (Wenzel *et al.*, 1991), de maneira bastante móvel e dinâmica (Haber *et al.*, 2006). Tal posição estratégica permite aos astrócitos atuarem como células especializadas em diferentes funções. De modo geral, a conexão com os vasos sanguíneos contribui para o seu papel na regulação do fluxo sanguíneo (Anderson & Nedergaard, 2003), bem como também promove suporte metabólico para os neurônios, através da captação de glicose do sangue e atua como fonte de lactato (Tsacopoulos & Magistretti, 1996; Magistretti & Pellerin, 1997; Magistretti, 2011). Astrócitos são conhecidos por liberarem fatores de crescimento *in vitro*, incluindo fator de crescimento neuronal (NGF), fator neurotrófico derivado do encéfalo (BDNF), neurotrofina-3 (NT-3) (Rudge *et al.*, 1992; Yoshida & Toya, 1997) e fator de crescimento de fibroblasto (Vaca & Wendt, 1992). Estas

moléculas são essenciais no controle da maturação e sobrevivência neuronal (Ojeda *et al.*, 2000). Astrócitos também atuam nos processos de neurogênese na fase adulta (Bordey, 2006; Lledo & Gheusi, 2006), e outros estudos já mostraram que astrócitos controlam a diferenciação neuronal *in vitro* através da liberação de fatores neurotróficos dependente de sua atividade (Blondel *et al.*, 2000).

Por outro lado, uma variedade de evidências do papel dos astrócitos sobre a transmissão sináptica está sendo sugerida, classificando-os tanto como “ouvintes” como também “falantes” das rotinas sinápticas. Primeiramente, astrócitos ajudam a controlar os níveis extracelulares de alguns íons, por exemplo, através da captação de K^+ (liberado durante excitação sináptica). Dessa forma, os astrócitos previnem a hiper-excitabilidade neuronal (Kuffler & Nicholls, 1966). Ainda, astrócitos são capazes de captar neurotransmissores da fenda sináptica e depois devolver aos neurônios (Mennerick & Zorumski, 1994; Gottfried *et al.*, 2002; Goubard *et al.*, 2011). Apesar de ainda ser considerada uma atividade passiva, a remoção do excesso de neurotransmissores do espaço extracelular permite uma codificação precisa e discreta dos sinais sinápticos e da neurotransmissão.

Diante das sinapses e ao contrário dos neurônios, astrócitos são células eletricamente silenciosas. Entretanto, podem mostrar aumentos nos níveis de Ca^{2+} intracelular como uma forma de excitabilidade espontânea (Parri *et al.*, 2001; Nett *et al.*, 2002; Parri & Crunelli, 2003) ou em resposta a ativação neuronal (Pasti *et al.*, 1997), principalmente pelo fato de que astrócitos expressam receptores para uma grande variedade de neurotransmissores (Condorelli *et al.*, 1999; Verkhratsky *et al.*, 2009a, 2009b; Lalo *et al.*, 2011). Este aumento de Ca^{2+} é parcialmente mediado pela liberação dos estoques intracelulares através da ativação do receptor inositol trifosfato (IP3) e dos canais de Ca^{2+} operados por estoque no citoplasma. Astrócitos formam sincícios celulares conectados por junções comunicantes (Scemes & Giaume, 2006;

Giaume, 2010) e as elevações de Ca^{2+} podem ser distribuídas completamente nesta rede de células por difusão de IP3/Ca^{2+} através das *gap-junctions*, bem como a liberação de ATP e ativação de receptores purinérgicos P2Y (Cornell-Bell *et al.*, 1990; Guthrie *et al.*, 1999; Kuga *et al.*, 2011), ou ainda liberação de outras substâncias neuroativas (Banker, 1980; Wagner *et al.*, 2006).

Considerando as funções dos astrócitos e o crescente número de questões sobre as consequências de seu processo de ativação tão particular, os próximos tópicos desta tese compreendem dois estudos que caracterizam funções predominantemente astrocíticas: i) a secreção da proteína S100B como consequência da ativação do sistema colinérgico dos astrócitos e ii) o uso de novas ferramentas que possam ajudar a entender melhor o papel da ativação do astrócito na transmissão sináptica.

1.3. Primeiro estudo

1.3.1. A proteína S100B

No SNC, os astrócitos são responsáveis pela maior parte da expressão da proteína S100B, além de serem capazes de promover a secreção dessa proteína de maneira regulada (Gonçalves *et al.*, 2008). A proteína S100B pertence à família de proteínas S100 ligantes de cálcio, e no encéfalo, está envolvida em diversas tarefas, intra e extracelulares (Donato, 2003; Donato *et al.*, 2009). São funções intracelulares: a inibição da fosforilação protéica (Pozdnyakov *et al.*, 1998; Scotto *et al.*, 1998), a regulação da atividade enzimática, modulação do citoesqueleto e regulação do crescimento e diferenciação celular (Sen & Belli, 2007).

A S100B no meio extracelular desempenha diversas outras atividades. Estudos *in vitro* mostraram efeitos tróficos desta proteína em concentrações baixas (picomolar), os quais incluem crescimento de neuritos (Kligman & Marshak, 1985), o aumento da sobrevivência neuronal durante o desenvolvimento e após o dano (Villarreal *et al.*, 2011; Sorci *et al.*, 2013), e a modulação da função sináptica, através da captação de glutamato (Donato, 2001). Ao contrário, concentrações altas (micromolar) podem causar produção de óxido nítrico (NO) pelos astrócitos vizinhos e microglia (Petrova *et al.*, 2000), ativação de cascatas apoptóticas mediadas pelo receptor de produtos finais de glicação avançada (RAGE) (Li *et al.*, 1998), entre outros fatores, levando a um quadro de disfunção e morte neuronal (Van Eldik & Wainwright, 2003).

É preciso notar, entretanto, que a combinação dos efeitos extracelulares quanto a concentração de S100B são dependentes do modelo biológico empregado no estudo (Serbinek *et al.*, 2010) e pouco se sabe da correlação destes valores de concentração de S100B *in vivo*. De qualquer modo, experimentos *in vitro* com culturas de células e investigações *in vivo* com modelos animais são contribuições muito relevantes que ajudam a entender o significado da S100B no encéfalo.

Sabe-se que a S100B extracelular trabalha como uma citocina neurotrófica, podendo ser classificada como marcador de dano cerebral em diversas patologias (Gonçalves *et al.*, 2008; de Souza *et al.*, 2009). Nesse sentido, a doença de Alzheimer (DA) é a doença neurodegenerativa mais frequentemente estudada (Sheng *et al.*, 1994; Peskind *et al.*, 2001; Leclerc *et al.*, 2010). Observou-se, por exemplo, uma redução nos níveis de S100B no fluido cerebroespinal de ratos submetidos a diferentes modelos de demência em nosso grupo (Vicente *et al.*, 2008; Rodrigues *et al.*, 2009; Costa *et al.*, 2012). Enquanto que outros estudos em humanos relataram altos níveis de S100B encontrados em estágios distintos da DA

(Rothermundt *et al.*, 2003; Gruden *et al.*, 2007). Apesar das diferenças, a integração entre biomarcadores de dano cerebral e protocolos baseados em evidências pode representar um grande avanço na manipulação dos prejuízos cerebrais agudos, e a proteína S100B parece ser uma boa candidata.

1.3.2. Da secreção da S100B

Apesar da variedade de estudos que descrevem as diferentes atividades da proteína S100B no SNC, pouco se sabe sobre o seu mecanismo de secreção. Na verdade, especula-se a ideia de um mecanismo comum de secreção que parece estar no aumento nos níveis de Ca^{2+} intracelular dos astrócitos, o que caracterizaria um tipo particular de ativação astrocítica e consequente liberação de fatores tróficos (Vaca & Wendt, 1992; Gürsoy & Büyükuysal, 2010). Este argumento foi demonstrado em células de glioblastoma humanos, no qual níveis intracelulares de Ca^{2+} e Zn^{2+} foram capazes de regular a secreção de S100B (Davey *et al.*, 2001a). Um outro trabalho também descreveu um aumento na secreção de S100B, em culturas primárias de astrócitos, através da modulação de Ca^{2+} do retículo endoplasmático após tratamento agudo com solvente DMSO (Leite *et al.*, 2010 não publicado). Através do estudo em fatias de hipocampo agudas, pôde-se observar um aumento na secreção da S100B a partir da modulação dos íons K^{+} e Ca^{2+} extracelulares (Nardin *et al.*, 2009). Também foi descrito o envolvimento de diversos sistemas neuronais no aumento da secreção de S100B *in vitro*, entre eles, o sistema dopaminérgico (Nardin *et al.*, 2011; Herrmann *et al.*, 2012), serotoninérgico (Manev & Manev, 2002; Tramontina *et al.*, 2008a), glutamatérgico (Goncalves *et al.*, 2002), entre outros. Estes estudos consideraram, principalmente, a influência das alternativas terapêuticas indicadas para o tratamento

de doenças neurodegenerativas, cada qual baseada em suas respectivas hipóteses neuronais.

Pelo menos até agora, todas estas evidências apontaram para um mecanismo de ativação astrocítica que altera os níveis de S100B no meio extracelular, entretanto ainda faltam mais estudos que possam indicar o exato mecanismo de liberação desta proteína.

1.3.3. O sistema colinérgico e seu envolvimento na secreção de S100B

A acetilcolina (ACh) é sintetizada principalmente nos neurônios colinérgicos a partir da atividade da colina acetiltransferase (ChAt), que reune colina, uma amina natural encontrada na bicamda lipídica das membranas celulares, e acetil-coenzima A, um tioéster usado nas reações metabólicas (Oda, 1999). A transmissão colinérgica é finalizada pela ação da enzima acetilcolinesterase (AChE), que catalisa a hidrólise da ACh em colina e ácido acético, um processo essencial que permite a restauração da atividade do neurônio colinérgico (Fernandez *et al.*, 1996). No SNC, o sistema colinérgico é o principal sistema de neurotransmissão associado às funções cognitivas, incluindo memória, atenção seletiva e processamento emocional (De Lacalle *et al.*, 1994; Niewiadomska *et al.*, 2011). Portanto, este sistema neuronal é frequentemente relacionado aos sintomas cognitivos observados nos casos de demência e no envelhecimento normal (Bartus *et al.*, 1982; Terry Jr. & Buccafusco, 2003; Schliebs & Arendt, 2011).

A perda de neurônios colinérgicos e suas propriedades funcionais (Davies & Maloney, 1976) levou a descrição da "hipótese colinérgica", onde se postulava que tal perda da função colinérgica no SNC contribuía significativamente para o declínio

cognitivo e prejuízo da memória (Bartus, 2000). Então, a restauração da função colinérgica deveria reduzir a severidade da perda cognitiva. Esta hipótese foi mantida pela evidência de que compostos químicos capazes de inibir a AChE mostraram efeitos positivos sobre a cognição de pacientes com DA (Birks, 2006; Hansen *et al.*, 2008). Apesar de ainda serem a estratégia terapêutica mais utilizada, anticolinesterásicos (IAChE) possuem limitações de uso, principalmente devido aos efeitos adversos periféricos e toxicidade hepática (Giacobini, 1994, 1997; Ma *et al.*, 2003), por isso os esforços em identificar novas substâncias.

Nesse sentido, a huperzina-A (HupA) é um exemplo bastante recente. HupA é um alcalóide extraído da planta chinesa *Huperzia serrata* (Wang *et al.*, 1986; Ma *et al.*, 2007). Estudos mostraram sua capacidade de atuar na inibição seletiva da AChE e observaram uma redução significativa do prejuízo cognitivo em modelos animais e em pacientes com DA na população chinesa, com pouca toxicidade (Wang *et al.*, 2006, 2009b). Além disso, o acúmulo de evidências aponta para os múltiplos efeitos neuroprotetores exercidos pela HupA, atuando através de diversos sítios moleculares, que não incluem necessariamente sua atividade inibitória sobre a AChE (Zhang & Tang, 2006; Hemendinger *et al.*, 2008; Wang *et al.*, 2008; Zhang *et al.*, 2008). Estes estudos sobre efeitos alternativos também se estendem para outros IAChEs, frequentemente utilizados na clínica para tratamento da DA e outros tipos de demência (Racchi *et al.*, 2004; Nordberg, 2006; Pepeu & Giovannini, 2009).

Apesar dos astrócitos expressarem AChE (Thullbery *et al.*, 2005; Bond *et al.*, 2006; Anderson *et al.*, 2008), os efeitos dos IAChE nestas células são muito pouco estudados. Sabe-se que IAChE podem exercer efeitos protetores nos astrócitos *in vitro* (Lahiri & Farlow, 1996; Han *et al.*, 2000). Além do conhecido papel protetor antioxidante da HupA (Zhao & Li, 1999; Wang & Tang, 2007), seu efeito no aumento da secreção de NGF em culturas de astrócitos primários também já foi demonstrado

(Tang *et al.*, 2005). De fato, com o crescente número de estudos que incluem a importância das funções dos astrócitos nas doenças neurodegenerativas, identificar os efeitos farmacológicos e bioquímicos das estratégias terapêuticas disponíveis nos astrócitos é de grande importância para a DA.

O sistema colinérgico está bastante distribuído no SNC e periférico. Os dois tipos básicos de receptores colinérgicos são muscarínicos e nicotínicos. Estes receptores são também expressos em diversos tipos celulares, incluindo as células endoteliais e células do sistema imune (Sharma & Vijayaraghavan, 2002; Fujii *et al.*, 2008; Wessler & Kirkpatrick, 2008). Receptores colinérgicos nicotínicos (nAChRs) fazem parte da super-família de canais iônicos ativados por ligantes e estão envolvidos em numerosas funções fisiológicas do encéfalo, incluindo funções cognitivas tais como aprendizado e memória (Levin *et al.*, 2006; Dani & Bertrand, 2007). Estes receptores são caracterizados por um variado número de subunidades distintas, que por sua vez, são encontrados em diferentes composições. No SNC de mamíferos, nAChRs são compostos pela reunião de 5 subunidades: homoméricas da subunidade α (α7 ou α9) ou, alternativamente, algumas combinações de subunidades α (α2-α6) e β (β2-β4) (Albuquerque *et al.*, 2009). Cada subunidade dos nAChRs possui propriedades farmacológicas e biofísicas diferentes, dependendo da sua composição (Jensen *et al.*, 2005). Frequentemente, ativação dos nAChRs permite o movimento dos íons Na^+, K^+ e Ca^{2+} através da membrana (Rathouz & Berg, 1994; Rathouz *et al.*, 1996) e, além disso, essa ativação já foi reconhecida por facilitar a sobrevivência celular diante de insultos tóxicos em neurônios (Bencherif, 2009; Akaike *et al.*, 2010; Kawamata *et al.*, 2011).

Embora se tenha dedicado muito empenho nos estudos sobre a expressão funcional dos receptores nicotínicos neuronais, em diversas populações de neurônios no encéfalo, pouco se sabe sobre sua expressão e relevância funcional nas

células não-neuronais, especialmente nos astrócitos. Alguns trabalhos já mostraram a expressão funcional destes receptores em culturas de astrócitos e microglia (Sharma & Vijayaraghavan, 2002; Shytle *et al.*, 2004), além de também caracterizarem o papel da nicotina na ativação astrocítica através do aumento nos níveis de Ca^{2+} intracelular e na modulação das correntes de membrana em culturas de astrócitos de ratos (Oikawa *et al.*, 2005a; Hernández-Morales & García-Colunga, 2009) e, mais recentemente, outros trabalhos reportaram respostas funcionais específicas do receptor nicotínico α7 (α7nAChRs) em astrócitos em fatias de hipocampo agudas (Shen & Yakel, 2009; Pirttimaki *et al.*, 2013).

1.4. Segundo estudo

1.4.1. Astrócitos e sua participação na transmissão sináptica

Foi mencionado anteriormente que, apesar dos astrócitos serem células eletricamente não excitáveis, podem responder a neurotransmissores através de elevações de Ca^{2+} intracelular (Nedergaard, 1994; Zorec *et al.*, 2012), e nos últimos anos, muitos estudos vem relatando que esta forma de ativação leva a liberação de transmissores neuroativos, e que por sua vez modulam a comunicação entre as células da glia e entre a glia e neurônios (Perea & Araque, 2007a; Agulhon *et al.*, 2008; Fiacco *et al.*, 2009a). Gliotransmissão é o termo que define esta liberação de transmissores regulada pelos astrócitos e tem sido uma área relevante da neurociência desde sua descoberta em 1994 (Parpura *et al.*, 1994). Este conceito é de grande importância funcional para se entender melhor como o encéfalo trabalha tanto em condições fisiológicas normais como patológicas (Halassa *et al.*, 2007a;

Halassa & Haydon, 2010). Entretanto, os sinais de Ca^{2+} necessários para a liberação de gliotransmissores, o mecanismo de liberação e, de fato, a existência real deste fenômeno ainda estão sendo altamente debatidos (Fiacco *et al.*, 2009b; Agulhon *et al.*, 2010; Hamilton & Attwell, 2010; Nedergaard & Verkhratsky, 2012).

Dentre os gliotransmissores estudados, os mais comuns são os neurotransmissores glutamato, GABA e ATP (Gordleeva *et al.*, 2012). D-serina, um modulador dos receptores NMDA, quando liberado no hipocampo pelo astrócito parece controlar a plasticidade sináptica dependente de NMDA (Henneberger *et al.*, 2010). Estudos recentes tem demonstrado que a gliotransmissão pode ser controlada por outros fatores, além do Ca^{2+}, tais como, fator de necrose tumoral (TNFalfa) (Santello *et al.*, 2011), interleucinas (Ren & Dubner, 2010) e pela produção de espécies reativas de oxigênio (Liu *et al.*, 2009). Para a liberação de gliotransmissores, muitos mecanismos são propostos, tais como, exocitose tipo neuronal, por transportadores (Calì *et al.*, 2009; Cali *et al.*, 2014), ou ainda, a liberação pode ser modulada através de canais iônicos regulados por volume (VRAC), como por exemplo, a bestrofina 1 (Park *et al.*, 2009; Lee *et al.*, 2010).

As consequências da gliotransmissão na transmissão sináptica estão se acumulando na literatura. Estudos no hipocampo mostraram que astrócitos são responsáveis por modular correntes neuronais lentas de entrada (SICs) através da ativação de receptores NMDA extrassinápticos (Fellin *et al.*, 2004), e correntes lentas de saída (SOCs), pela ativação de receptores GABA-A (Le Meur *et al.*, 2012). Estas correntes parecem ocorrer na ausência da atividade sináptica neuronal e são observadas em diferentes áreas do hipocampo: CA1, CA3 e giro denteado. Outras evidências já reportaram o aumento da taxa de captação de K^+ devido as elevações intracelulares de Ca^{2+} nos astrócitos, promovendo dessa maneira uma modulação na atividade da rede neuronal no hipocampo (Wang *et al.*, 2012a) e na estabilidade das

células de Purkinje no cerebelo (Wang *et al.*, 2012b). Astrócitos também parecem influenciar a plasticidade cortical através da transformação de *inputs* colinérgicos *in vivo* (Takata *et al.*, 2011). Recentemente, mostrou-se que a ativação do receptor canabinóide CB1 induziu nos astrócitos a liberação de glutamato gerando LTD (depressão de longa duração) no hipocampo (Han *et al.*, 2012) e córtex (Min & Nevian, 2012).

Apesar do número crescente de estudos, os mecanismos da liberação de gliotransmissores ainda não está esclarecido e um dos obstáculos existentes que desafiam os pesquisadores está no fato de que astrócitos expressam muitos receptores, os quais também são presentes em neurônios (Fiacco *et al.*, 2009a), e que ainda não foi possível desenvolver uma metodologia específica, capaz de estimular os astrócitos seletivamente.

1.4.2. Recentes metodologias para entender a gliotransmissão

Nos últimos anos, novos métodos foram desenvolvidos para ativar astrócitos especificamente. Primeiro, foram gerados camundongos transgênicos expressando um receptor acoplado à proteína G (GPCR), normalmente encontrado no sistema nervoso periférico, em astrócitos (Fiacco *et al.*, 2007; Agulhon *et al.*, 2010). A ativação específica dos íons Ca^{2+} nestes camundongos não teve efeito sobre a plasticidade ou transmissão sináptica ou ainda, sobre a excitação neuronal, sugerindo que o aumento dos níveis de Ca^{2+} em astrócitos não era suficiente para liberar gliotransmissores (Shigetomi *et al.*, 2008, 2010). Estes resultados contradiziam outros estudos que mostravam a gliotransmissão dependente de Ca^{2+} (Andersson *et al.*, 2007; Perea & Araque, 2007b; Halassa & Haydon, 2010). Outras estratégias

genéticas foram desenvolvidas, baseadas em *knockouts* para proteínas específicas dos astrócitos. Por exemplo, para se estudar a liberação de gliotransmissores por exocitose, pesquisadores desenvolveram camundongos que expressavam um dominante negativo para a proteína SNARE (sob um promotor para GFAP) (Pascual *et al.*, 2005). A interrupção da sinalização de Ca^{2+} intracelular, específica para astrócitos, através da desativação genética dos receptores IP3 também foi usada para investigar a influência do Ca^{2+} intracelular sobre neurônios (Petravicz *et al.*, 2008).

Recentemente, uma alternativa que se apresenta interessante é o uso de ferramentas em optogenética. Canais iônicos ativados por luz são rotineiramente utilizados para ativar neurônios de modo pouco invasivo e com alta resolução espaço-temporal (Gradinaru *et al.*, 2009; Wyart *et al.*, 2009; Szobota & Isacoff, 2010).

Dentre os canais já utilizados, destaca-se canal-rodopsina 2 (ChR2) (Nagel *et al.*, 2003), primeiramente introduzida nos estudos em neurônios de mamíferos (Boyden *et al.*, 2005), e depois também utilizada para ativar respostas neuronais dependentes dos astrócitos *in vivo* (Gradinaru *et al.*, 2009; Gourine *et al.*, 2010). Entretanto, experimentos em culturas de astrócitos mostraram que devido a sua baixa permeabilidade ao Ca^{2+} e rápida inativação, ChR2 não pôde evocar elevações de Ca^{2+} consistentes em astrócitos (Nagel *et al.*, 2003; Lin *et al.*, 2009), os quais, diferentemente dos neurônios, não possuem canais de Ca^{2+} ativados por voltagem (Carmignoto *et al.*, 1998; Parpura & Verkhratsky, 2012a). Outros canais vem sendo testados: o primeiro é derivado do receptor ionotrópico de glutamato do tipo 6, receptor de glutamato ativado por luz permeável ao cálcio (LiGluR) (Volgraf *et al.*, 2006; Szobota & Isacoff, 2010) e uma variação do canal-rodopsina translocador de Ca^{2+} (CatCh) (Kleinlogel *et al.*, 2011). Ambos evocaram sinais de Ca^{2+} confiáveis e robustos em astrócitos (Li *et al.*, 2012a). A ativação do canal LiGluR mostrou uma modulação na entrada direta de Ca^{2+} através da membrana plasmática, que ainda é

posteriormente moldada pelos estoques de Ca^{2+} internos. Enquanto que a ativação de CatCh gerou uma entrada de Ca^{2+} independente da depleção dos estoques de Ca^{2+} internos, indicando que os dois canais são ferramentas interessantes capazes de ativar vias de sinalização de Ca^{2+} de modo seletivo e distinto (Li *et al.*, 2013).

A expressão específica destes canais ativados por luz em astrócitos pode ser realizada a partir de diversos sistemas. Neste estudo, foi testado aquele que combina sistemas de vetores virais (Royo *et al.*, 2007), que carregam um constructo de interesse específico, e animais transgênicos do tipo cre-recombinase ($CreER^{T2}$), desenvolvidos sob a orientação de um promotor conhecidamente específico para os astrócitos (Mori *et al.*, 2006). O sucesso desta etapa de caracterização viral, e a verificação do bom funcionamento destes canais na ativação dos astrócitos são de extrema relevância para o avanço deste estudo em gliotransmissão. Se neurônios e astrócitos compartilham os mesmos receptores e moléculas transmissoras, fica difícil diferenciar entre o efeito direto sobre o sinal de transmissão neuronal e um efeito sobre os neurônios, causado pela ativação dos astrócitos e a subsequente gliotransmissão sentida pelos neurônios. Entretanto, existem limitações que não podem deixar de serem consideradas. Dentre os principais desafios, destacam-se a falta de especificidade para a expressão das proteínas de interesse e uma possível astrogliose reativa, decorrente da injeção estereotáxica do vírus, ou do vírus propriamente dito.

2. Objetivos

2.1. Gerais

Investigar ativação astroglial em dois modelos distintos, avaliando secreção da proteína S100B e liberação de gliotransmissores.

2.2. Específicos

Quanto ao primeiro estudo (capítulo 1) em culturas primárias de astrócitos:

I. Verificar os efeitos dos anticolinesterásicos, HupA e tacrina, sobre a secreção de S100B;

II. Investigar se agonistas colinérgicos, tanto muscarínicos como nicotínicos, são capazes de alterar a secreção de S100B;

Quanto ao segundo estudo (capítulo 2):

I. Caracterizar a expressão específica e funcional de canais iônicos ativados por luz em astrócitos na região CA1 do hipocampo através do uso de sistemas de distribuição viral combinados a animais transgênicos do tipo cre-recombinase induzível;

II. Manipular a atividade de astrócitos em fatias hipocampais agudas através do uso de ferramentas em optogenética e estudar as consequências destas manipulações específicas na geração de correntes lentas em neurônios.

PARTE II

Capítulo 1

Huperzine A, but not tacrine, stimulates S100B secretion in astrocyte cultures.

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Huperzine A, but not tacrine, stimulates S100B secretion in astrocyte cultures

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ABSTRACT

Aims: The loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and dementias. Huperzine A (HupA) is a selective inhibitor of acetylcholinesterase (AChE) and has been shown to significantly reduce cognitive impairment in animal models of dementia. Based on the importance of astrocytes in physiological and pathological brain activities, we investigated the effect of HupA and tacrine on S100B secretion in primary astrocyte cultures. S100B is an astrocyte-derived protein that has been proposed to be a marker of brain injury.
Main methods: Primary astrocyte cultures were exposed to HupA, tacrine, cholinergic agonists, and S100B secretion was measured by enzyme-linked immunosorbent assay (ELISA) at 1 and 24 h.
Key findings: HupA, but not tacrine, at 100 μM significantly increased S100B secretion in astrocyte cultures. Nicotine (at 100 and 1000 μM) was able to stimulate S100B secretion in astrocyte cultures.
Significance: Our data reinforce the idea that AChE inhibitors, particularly HupA, do not act exclusively on the acetylcholine balance. This effect of HupA could contribute to improve the cognitive deficit observed in patients, which are attributed to cholinergic dysfunction. In addition, for the first time, to our knowledge, these data indicate that S100B secretion can be modulated by nicotinic receptors, in addition to glutamate, dopamine and serotonin receptors.

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Introduction

Huperzine A (HupA), a Lycopodium alkaloid isolated from the Chinese herb **Huperzia serrata**, (Ma et al., 2007; Wang et al., 1986) is a selective inhibitor of acetylcholinesterase (AChE) and has been shown to significantly reduce cognitive impairment in animal models of dementia and in Alzheimer's disease (AD) patients in China (Wang et al., 2006, 2009). Compared with other AChE inhibitors, e.g. tacrine, HupA demonstrates a better passage through the blood–brain barrier, higher oral bioavailability, longer duration of AChE inhibition, fewer peripheral side effects, and is devoid of dose-limiting hepatotoxicity (Bai et al., 2000; Liang and Tang, 2004; Tang, 1996; Tang et al., 1994; Wang and Tang, 1998a; Xiong and Tang, 1995).

AD exhibits several neurochemical alterations including β-peptide amyloidosis, inflammation and oxidative stress, leading to neuronal population destruction, especially of cholinergic neurons (Davies and Maloney, 1976; Geula et al., 2008; Mufson et al., 2002, 2008; Verdier et al., 2004). This loss of cholinergic function in the central nervous system contributes significantly to the cognitive decline associated with advanced age and AD (Bartus, 2000; Bartus et al., 1982; Schliebs and Arendt, 2011; Terry and Buccafusco, 2003) and has justified the use of AChE inhibitors as a strategy for patients with AD (Birks, 2006; Giacobini, 2000; Nordberg and Svensson, 1998; Talesa, 2001; Whitehouse, 1993). Moreover, many other neuroprotective effects of these compounds, in addition to AChE inhibition, have been described (Nordberg, 2006; Racchi et al., 2004; Takatori, 2006). Some studies have found that HupA displays neuroprotective properties with multi-target effects (Zhang and Tang, 2006; Zhang et al., 2008a, 2008b). HupA has improved chronic inflammation and cognitive decline in diverse animal models of dementia (Wang et al., 2001, 2010; Wang and Tang, 1998b; Zhang et al., 2004; Zhou et al., 2001).

S100B, a calcium-binding protein, produced and secreted by astrocytes in the central nervous system, plays a regulatory role in the cytoskeleton and cell cycle (Donato et al., 2009). Extracellular S100B works as a neurotrophic cytokine and has been proposed as a marker for brain injury, including that seen in AD (Goncalves et al., 2008). In fact, it has been reported that this protein could affect neuronal β-amyloid protein synthesis (Li et al., 1998). On the other hand, we have observed decreased levels of this protein in the cerebrospinal fluid in rat models of dementia, including chronic cerebral hypoperfusion (Vicente et al., 2009), intracerebroventricular administration of streptozotocin (Rodrigues et al., 2009) and okadaic acid (Costa et al., 2012).

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S100B secretion appears to be modulated by antipsychotic (Nardin et al., 2011; Steiner et al., 2009) and anti-depressive drugs (Manev and Manev, 2002; Tramontina et al., 2008), commonly used for the symptomatic treatment of dementia. However, the effect of AChE inhibitors and cholinergic agonists on S100B secretion has not been investigated. Considering the importance of astrocytes in physiological and pathological brain activities (Halassa et al., 2009; Halassa and Haydon, 2010), we decided to investigate the effect of HupA and tacrine on S100B secretion astrocyte cultures using HupA at concentrations currently described in the literature (from 0.1 to 100 μM), as well as cholinergic agonists. Our results suggest, for the first time to our knowledge, a cholinergic modulation of S100B secretion.

Material and methods

Animals

Twenty newborn Wistar rats (1 or 2-days old) (used for astrocyte cultures) and twelve male 30-day old rats (used for hippocampal slice preparations) were obtained from our breeding colony (at the Department of Biochemistry, Universidade Federal do Rio Grande do Sul, Brazil). Rats were maintained under controlled light and environmental conditions (12 h light/12 h dark cycle at a constant temperature of 22 ± 1 °C) with free access to food and water. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23), revised 1996, and following the regulations of the local animal house authorities (Approval 20375).

Drugs and reagents

(−)Huperzine A, tacrine, acetylthiocholine, nicotine, mecamylamine, carbachol, scopolamine, poly-L-lysine, monoclonal anti-S100B antibody (SHB1), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), **o**-phenylenediamine (OPD) and methylthiazolyldiphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, USA). Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM) and other materials for cell culture were purchased from Gibco (Carlsbad, USA). Polyclonal anti-S100B and anti-rabbit linked peroxidase were purchased from DAKO (São Paulo, Brazil) and GE (Little Chalfont, United Kingdom), respectively.

Astrocyte cultures

Primary astrocyte cultures from Wistar rats were prepared as previously described (Gottfried et al., 1999). Briefly, cerebral cortices of newborn Wistar rats were removed and mechanically dissociated in Ca^{2+}- and Mg^{2+}-free balanced salt solution, pH 7.4, containing (in mM): 137 NaCl; 5.36 KCl; 0.27 Na_2HPO_4; 1.1 KH_2PO_4 and 6.1 glucose. The cortices were cleaned of meninges and mechanically dissociated by sequential passages through a Pasteur pipette. After centrifugation at 1400 RPM for 5 min, the pellet was resuspended in DMEM (pH 7.6) supplemented with 8.39 mM HEPES, 23.8 mM $NaHCO_3$, 0.1% amphotericin, 0.032% gentamicin and 10% fetal calf serum. The cells were plated at a density of 1.5×10^5 cells/cm^2 in 24 well plates pre-coated with poly-L-lysine. Cultures were maintained in DMEM containing 10% FCS in 5% CO2/95% air at 37 °C, allowed to grow to confluence, and used in vitro at 15 days. Immunocytochemistry for glial fibrillary acidic protein (GFAP), a specific protein for astrocytes, indicated a cell purity of higher than 95% (data not shown). Treatments consisted of HupA (0.1, 1, 10 and 100 μM), tacrine (10 and 100 μM), acetylthiocholine (10 and 100 μM), nicotine, mecamylamine, carbachol and scopolamine, all these last four drugs were used at 1–1000 μM, for 1 and 24 h at 37 °C. Drug concentrations were chosen based on pilot experiments. All experiments were performed in triplicate. Afterwards, the collected culture medium and the cell lysates were stored at −20 °C until used in assays for S100B.

Hippocampal slices

Hippocampal slices were prepared as previously described (Nardin et al., 2009). Thirty-day old Wistar rats were killed by decapitation and the brains were removed and placed in cold saline medium with the following composition (in mM): 120 NaCl; 2 KCl; 1 $CaCl_2$; 1 $MgSO_4$; 25 HEPES; 1 KH_2PO_4, and 10 glucose, adjusted to pH 7.4 and previously aerated with O_2. The hippocampi were dissected and transverse slices of 0.3 mm were obtained using a McIlwain Tissue Chopper. Slices were then transferred immediately to 24-well culture plates, each well containing 0.3 ml of physiological medium and only one slice. The medium was changed every 15 min for 2 h at room temperature. After this stabilization period, the drugs were added and the slices incubated for 1 h at 30 °C. Treatments were the same as those used in astrocyte cultures.

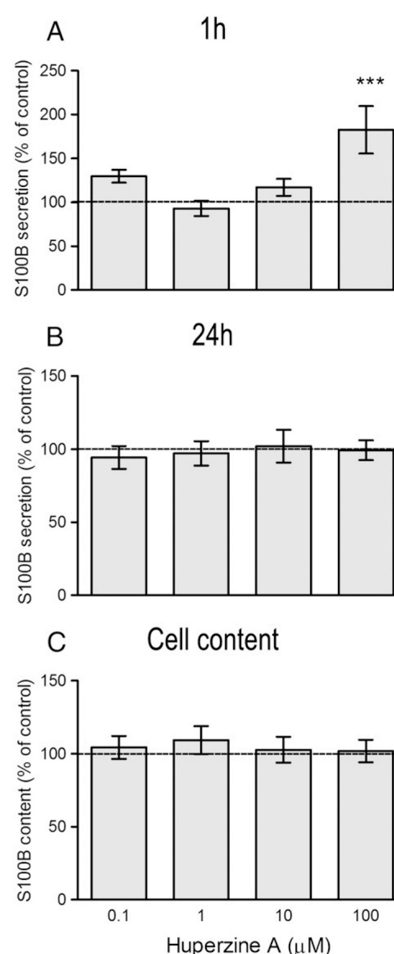

Fig. 1. *Effect of Huperzine A (HupA) on S100B secretion and content*. Astrocyte cultures were incubated with HupA (0.1, 1, 10 and 100 μM) and the S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate, expressed as a percentage of the control (indicated by dashed line). ***$p < 0.001$ compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

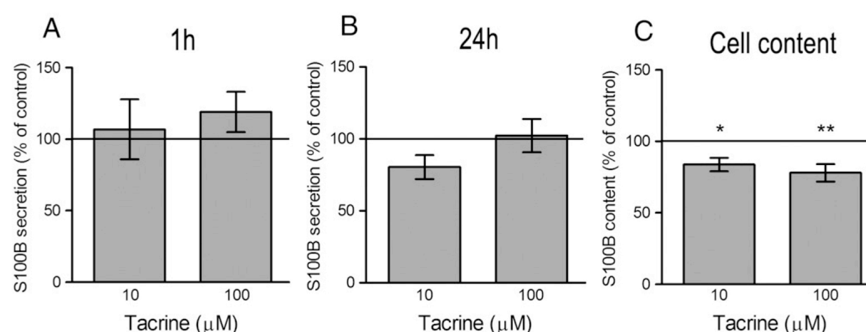

Fig. 2. *Effect of tacrine on S100B secretion and content.* Astrocyte cultures were incubated with tacrine (10 and 100 μM). S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate, expressed as a percentage of the control (indicated by line). *p<0.05 and **p<0.01 compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

S100B measurement

S100B levels were measured by enzyme-linked immunosorbent assay (ELISA), as previously described (Leite et al., 2008). Briefly, 50 μl of sample plus 50 μl of Tris buffer were incubated for 2 h in a microtiter plate previously coated with monoclonal anti-S100B. Polyclonal anti-S100 was incubated for 30 min and then peroxidase-conjugated anti-rabbit antibody was added for a further 30 min. The color reaction with **o**-phenylenediamine was measured at 492 nm. The standard S100B curve ranged from 0.002 to 1 ng/ml.

Cell viability and integrity

Cell viability was assayed by the colorimetric MTT reduction method (Hansen et al., 1989) and by neutral red incorporation (Leite et al., 2009). For the MTT reduction, cells were incubated with 0.5 mg/ml of MTT at 37 °C for 30 min. The formazan product generated during the incubation was solubilized in dimethyl sulfoxide (DMSO). Absorbance values were measured at 560 and 630 nm. The neutral red assay was carried out by treating cells with 50 μg/ml neutral red (NR) at 37 °C for 30 min. Afterwards, the cells were rinsed twice with PBS for 5 min each and NR dye taken up by viable cells was extracted with acetic acid/ethanol/water (1/50/49). Absorbance values were measured at 560 nm. Cell integrity was indicated by lactate dehydrogenase (LDH) activity in the incubation medium. Determination was carried out by a colorimetric commercial kit (Doles Reagentes e Equipamentos para Laboratórios Ltda., Brazil), according to the manufacturer's instructions. Results were expressed as percentages of the control.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dunnet's post hoc test. Results represent mean ± S.E.M. GraphPad Prism 5 for Windows was used for statistical analysis; p<0.05 was set as statistically significant, and values of significance were indicated by different symbols: one asterisk (*) for p<0.05; two asterisks (**) for p<0.01; and three asterisks (***) for p<0.001.

Table 1
Viability and integrity of astrocyte cultures and hippocampal slices treated with HupA, at 10 or 100 μM, for 24 h and 1 h, respectively.

	Astrocyte cultures		Hippocampal slices	
	NR	LDH	NR	LDH
HupA (10 μM)	100.0 ± 7.7	114.2 ± 9.3	119.2 ± 1.3	99.0 ± 7.3
HupA (100 μM)	110.3 ± 9.5	105.8 ± 7.0	123.1 ± 4.8	94.1 ± 1.4

NR, neutral red uptake; LDH, lactate dehydrogenase activity. Data are expressed as percentage of control and represent mean ± S.E.M.

Results

Huperzine A, but not tacrine, stimulates S100B secretion in astrocyte cultures

We investigated S100B secretion in astrocyte cultures by adding concentrations of HupA from 0.1 to 100 μM (Fig. 1A). HupA at 100 μM induced an increase in S100B secretion at 1 h ($F_{4,\ 25}=6.608$, $p=0.0009$). However, no changes were observed in secretion (Fig. 1B) or in the intracellular S100B content (Fig. 1C) at 24 h afterwards. In contrast, tacrine at 10 or 100 μM was not able to alter S100B secretion at 1 h (Fig. 2A) and at 24 h (Fig. 2B). Nevertheless, tacrine at both concentrations was able to reduce the intracellular content of S100B at 24 h (Fig. 2C) ($F_{2,\ 14}=7.058$, $p=0.0076$).

Huperzine A exposure did not affect cell integrity or viability

In order to evaluate cell integrity and viability of astrocytes exposed to HupA for 24 h, at two higher concentrations (10 and 100 μM), we carried out 3 assays: LDH activity, neutral red assay and MTT reduction assay. No significant differences were observed in extracellular LDH activity, suggesting that cell integrity was preserved during HupA exposure (Table 1). Two other assays for cell viability, neutral red assay (Table 1) and MTT reduction assay (data not shown), were not modified by HupA.

Acetylthiocholine, like Huperzine A, stimulated S100B secretion in astrocyte cultures

Acetylthiocholine is the substrate used for measurement of AChE activity and also works as a cholinergic agonist. This compound, at 100 μM, increased S100B secretion in astrocyte cultures at 1 h (Fig. 3A) ($F_{2,\ 13}=21.04$, $p=0.0001$). No changes in S100B secretion (Fig. 3B) or S100B content (Fig. 3C) were observed at 24 h.

Cholinergic modulation of S100B secretion in astrocyte cultures

Results for tacrine and acetylthiocholine suggest that the effect of HupA on S100B secretion was independent of AChE inhibition and possibly involves cholinergic regulation. Therefore, we investigated the involvement of cholinergic modulation in basal S100B secretion in astrocyte cultures. Four compounds were used: nicotine, mecamylamine (a nicotinic antagonist), carbachol (a muscarinic agonist) and scopolamine (a muscarinic antagonist), at concentrations from 1 to 1000 μM.

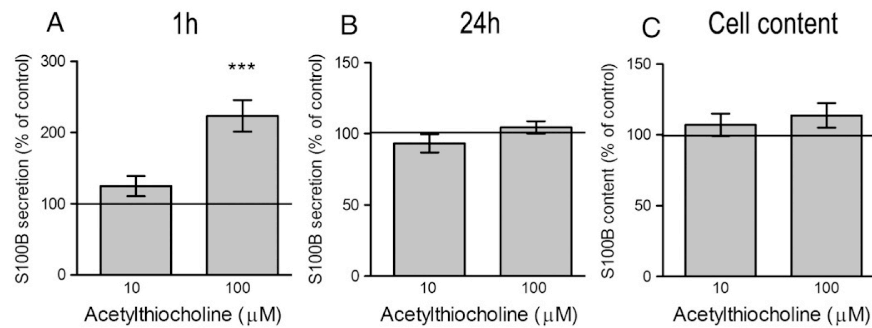

Fig. 3. *Effect of acetylthiocholine on S100B secretion and content*. Astrocyte cultures were incubated with acetylthiocholine (10 and 100 μM). S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate, expressed as a percentage of the control (indicated by line). ***p<0.001 compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

When nicotine at 100 or 1000 μM was added to astrocyte cultures (Fig. 4A), we observed an increase in S100B secretion at 1 h ($F_{4,\ 46}=$ 5.134, $p=0.0017$). No changes were observed in S100B secretion (Fig. 4B) or S100B intracellular content (Fig. 4C) at 24 h. Expectedly, mecamylamine was not able to change S100B secretion at 1 h (Fig. 5A), but increased S100B secretion at 24 h (Fig. 5B) ($F_{4,\ 22}=$ 12.30, $p=0.0001$). No changes, induced by mecamylamine, were observed in the S100B intracellular content (data not shown). Mecamylamine (at 1 mM) blocked the increase in S100B secretion induced by HupA, and also blocked the effect of nicotine (Fig. 5C) ($F_{3,\ 33}=9.519$, $p=0.0001$). No changes in S100B secretion or content were observed when carbachol or scopolamine was added to astrocyte cultures (data not shown).

S100B secretion was reduced by Huperzine A and acetylthiocholine in fresh hippocampal slices

Finally we evaluated the effect of HupA on basal S100B secretion in acute hippocampal slices. Surprisingly, HupA at 100 μM decreased S100B secretion in this preparation (Fig. 6A) ($F_{4,\ 53}=5.165$, $p=$ 0.0014). This effect also occurred with acetylthiocholine at 100 μM (Fig. 6B, $F_{2,\ 22}=12.51$, $p=0.0002$), but not with tacrine at 100 μM (data not shown). Nicotine at 100 and 1000 μM (Fig. 6C) did not significantly change S100B secretion, but at lower concentrations (1 and 10 μM) we observed an increase in S100B secretion ($F_{4,\ 37}=3.628$, $p=$ 0.0136).

Discussion

HupA has been used in schizophrenia and AD in China and has been proposed as a selective, reversible, and well-tolerated inhibitor of AChE, and an even more potent inhibitor of AChE than tacrine, donepezil, rivastigmine or galanthamine in vivo (Ma et al., 2007; Wang et al., 2009). However, most of the clinical trials that have employed this compound have been performed in China and this substance has not been approved by the Food and Drug Administration (FDA) in the USA or by the National Agency of Sanitary Vigilance (ANVISA) in Brazil yet. As such, the characterization of some putative effects of this compound on some neurochemical targets is necessary.

We herein analyzed the effect of HupA on S100B secretion, assumed to be a marker of activation of astrocytes (Donato et al., 2009; Goncalves et al., 2008), which play an important role in both physiological and pathological brain processes (Halassa and Haydon, 2010; Rodriguez et al., 2009; Verkhratsky, 2006). Recent studies have emphasized the role of astrocytes (via nicotinic and muscarinic receptors) in cholinergic neurotransmission and long-term potentiation (LTP) (Navarrete et al., 2012; Shen and Yakel, 2012). Moreover, a role of extracellular S100B in LTP has been previously reported (Nishiyama et al., 2002).

Our results indicate that HupA is able to induce an increase in S100B secretion in cortical astrocytes in culture, but a decrease in S100B secretion in acute hippocampal slices. These opposing effects

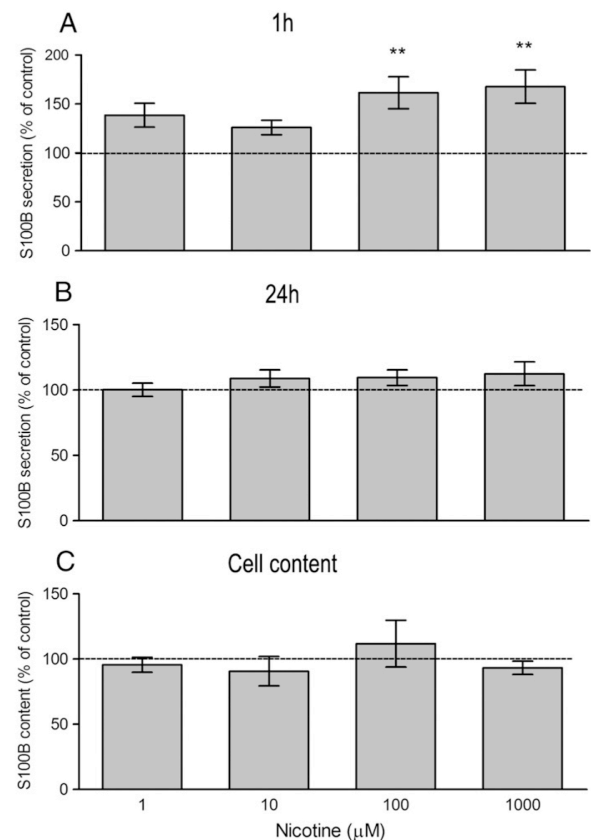

Fig. 4. *Effect of nicotine-induced S100B secretion and content*. Astrocyte cultures were incubated with nicotine (1, 10, 100 and 1000 μM) and the S100B levels in the medium were measured for 1 h (A) and 24 h (B), as well as the S100B content (C) by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate and expressed as a percentage of the control (indicated by dashed line). **p<0.01 compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

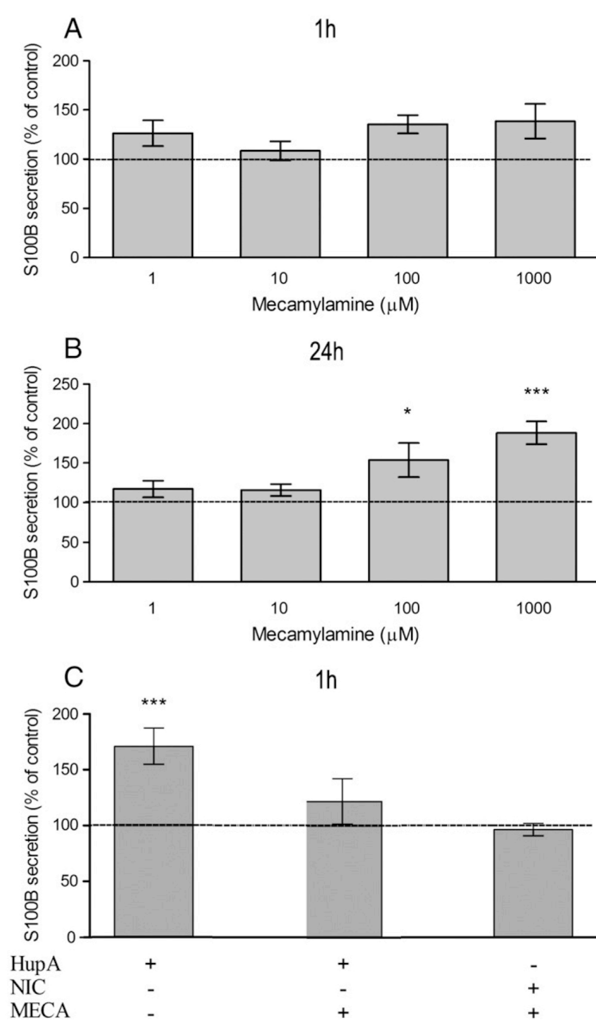

Fig. 5. *Effect of mecamylamine-induced S100B secretion and content*. Astrocyte cultures were incubated with mecamylamine (1, 10, 100 and 1000 μM) and the S100B levels in the medium were measured for 1 h (A) and 24 h (B). In C, astrocyte cultures were incubated with mecamylamine (MECA, at 1 mM) and nicotine (NIC, at 1 mM) or Huperzine A (HupA, at 100 μM) for 1 h. S100B levels in the medium were measured by ELISA. Each value is the mean (± standard error) of five independent experiments performed in triplicate and expressed as a percentage of the control (indicated by dashed line). *p<0.05 and ***p<0.001 compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

have also been described in other studies; for example, endothelin-1 induces S100B secretion in astrocyte cultures, but a decrease in hippocampal slices (Leite et al., 2009). Such discrepancies can be attributed to differences in cell signaling between isolated cells and the complex cell environment of slice preparation and/or the heterogeneity of astrocyte populations from different brain regions. Moreover, under our conditions, no differences were found in relation to cell integrity or viability in either preparation.

Our results regarding the increase in S100B secretion in cortical astrocytes suggest a trophic role of HupA, based on the in vitro neurotrophic activity of S100B (Donato et al., 2009; Serbinek et al., 2010) and based on the acute administration of this protein in vivo after brain injury (Kleindienst and Ross Bullock, 2006). In contrast, tacrine was not able to induce S100B secretion in astrocyte cultures (or a decrease in hippocampal slices). This result suggests that the modulation of S100B secretion by HupA is independent of AChE inhibition, but does not rule out a possible effect of HupA on cholinergic neurotransmission.

Confirming this possibility, a similar effect to that of HupA on S100B secretion was also obtained with acetylthiocholine, a cholinergic agonist, in both cortical astrocytes and hippocampal slices. Nicotine was also able to induce S100B secretion in astrocyte cultures (at concentrations of 100 and 1000 μM) and in hippocampal slices (at concentrations of 1 and 10 μM). The Huperzine A or nicotine-induced S100B secretion in astrocyte cultures was blocked by mecamylamine, a non-selective nicotinic antagonist. Moreover, in astrocyte cultures mecamylamine did not affect basal S100B secretion at 1 h, but was able to increase S100B secretion 24 h later. No effect on acute basal S100B secretion was observed with the addition of carbachol, a muscarinic agonist, or scopolamine, a muscarinic antagonist. Therefore, these data suggest a specific involvement of nicotinic receptors in the modulation of S100B secretion in astrocyte cultures.

The mechanism by which HupA affects S100B secretion remains unclear, but we may make some speculations regarding the involvement of cholinergic and/or NMDA receptors. Studies on the displacement of $[H^3]$QNB- and $[H^3](-)$nicotine-specific binding showed that HupA has a direct effect on cholinergic receptors (Tang et al., 1989). However, there are no data about binding or the direct action of HupA on cholinergic presynaptic receptors and the control of acetylcholine release (Wang et al., 2006). On the other hand, HupA was able to block the release of TNF-alpha, an inflammatory cytokine, in cultures of microglial cells submitted to hypoxia, and this effect was antagonized by mecamylamine (Wang et al., 2012). In agreement with this, our data reinforce the involvement of nicotinic receptors in HupA activity. Moreover, it has been proposed that HupA acts as an NMDA antagonist (Gordon et al., 2001). This possibly should be investigated, however NMDA was unable to induce S100B secretion in astrocyte cultures (Goncalves et al., 2002).

As mentioned before HupA has also been used in the treatment of schizophrenia in China (Ma et al., 2007). Antipsychotic drugs (putatively acting on dopamine receptors) are reported to modulate S100B secretion (Nardin et al., 2011; Steiner et al., 2009). Therefore, it would be interesting to evaluate whether the modulation of S100B secretion by HupA also involves dopamine receptors.

Conclusions

Our data reinforce the idea that AChE inhibitors, particularly HupA, do not act exclusively on the acetylcholine balance. HupA, per se, could be acting via nicotinic receptors, at least with regards to astroglial S100B secretion. This action could contribute to improve the cognitive deficit observed in AD patients, which is attributed to cholinergic dysfunction. This reinforces the importance of astrocytes as targets of neuroactive compounds in brain diseases. In addition, for the first time, to our knowledge, these data indicate that S100B secretion can be modulated by nicotinic receptors, in addition to glutamate, dopamine and serotonin receptors.

Conflict of interest statement

There is no conflict of interest.

Acknowledgments

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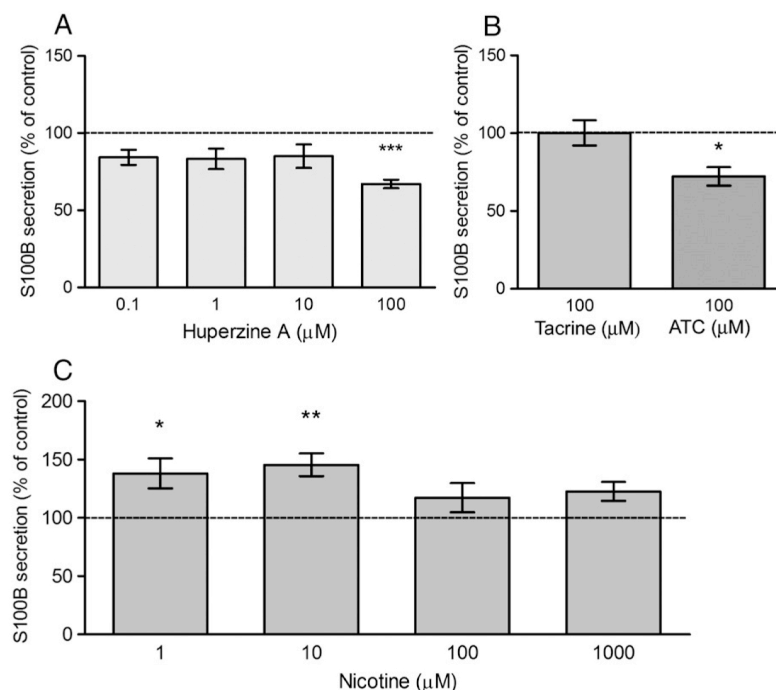

Fig. 6. *Effect of Huperzine A, tacrine, acetylthiocholine and nicotine on S100B secretion in hippocampal slices.* Hippocampal slices were incubated with HupA (0.1, 1, 10 and 100 μM) (A), tacrine and acetylthiocholine (ATC) at 100 μM (B) and nicotine (1, 10, 100 and 1000 μM) (C). S100B levels in the medium were measured for 1 h by ELISA. Each value is the mean (± standard error) of six independent experiments performed in triplicate, expressed as a percentage of the control (indicated by dashed line). *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to control (one-way ANOVA followed by Dunnet's post-hoc assay).

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Capítulo 2

Optogenetic investigation of gliotransmission in brain tissue.

Artigo em preparação.

Optogenetic investigation of gliotransmission in brain tissue.

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Abstract

Brain tissue includes two main cell types, neurons and glia. Neurons are viewed to be the information-processing cells. However, accumulative evidence indicates that glial cells can directly and dynamically regulate neuronal activity by releasing neuro-active molecules, named gliotransmitters. Within glia, astrocytes occupy are majority cells of the grey matter and form a heterogeneous cell population. About ten years ago it was suggested that in addition to their supportive role, astrocytes contribute actively to information processing by regulating synaptic activity leading to the concept of the *tripartite synapse,* which includes the neuronal pre- and post-synaptic elements and an astrocyte process, all interacting in a reciprocal manner. Astrocytes respond to neuronal activity with cytosolic calcium elevations that, in turn, induce the release of neuroactive gliotransmitters from astrocytes. These gliotransmitters can stimulate other astrocytes and modify the excitability of neighboring pre- and post-synaptic elements. The aim of this work was to manipulate the activity of astrocytes in hippocampal slices through optogenetic tools and to evaluate the consequences of these specific manipulations on the generation of NMDA receptor- and GABA receptor-mediated currents in neurons recorded by means of the patch clamp technique. Here, we reviewed the importance of gliotransmission studies and we reported the characterization of a viral approach to target astrocytes. Since targeting astrocytes was a crucial step of this work, a discussion about hope and pitfalls can be relevant to scientific community.

Keywords: astrocytes, gliotransmission, calcium, adeno-associated virus, and immunohistochemistry.

Introduction

Astrocytes occupy about 50% of the grey matter in the human cortex. They are ensheathing neuronal synapses with their processes and the brain vasculature with their end feet (Iadecola & Nedergaard, 2007; Rossi *et al.*, 2007). This strategic position allows them to play several important roles in the brain. Their close connections to the blood vessels contribute to their role for regulation of blood flow (Anderson & Nedergaard, 2003) as well as for metabolic support of neurons through uptake of glucose from the blood and supply of lactate to neurons (Tsacopoulos & Magistretti, 1996; Magistretti & Pellerin, 1997; Magistretti, 2011). On the other hand they have multiple influences on synaptic transmission allowed by their dynamic enwrapping of synapses. Their first accepted function for signaling in brain was the spatial K^+ buffering. During neuronal excitation K^+ is released in the extracellular space. Astrocytes prevent neuronal hyper excitability through uptake of K^+ with K^+ inward rectifying channels and distribution over the astrocytic syncytium (Kuffler & Nicholls, 1966). Additionally, they are taking up neurotransmitters from synaptic cleft and reprovide them to neurons (Mennerick & Zorumski, 1994).

Astrocytes are, in contrary to neurons, electrically silent, but show increases in their intracellular calcium (Ca^{2+}) concentration as a form of excitability in response to neuronal activation. The Ca^{2+} increase is partly mediated by release from the intracellular stores through inositol triphosphate (IP_3) receptor activation and store-operated Ca^{2+}-channels in the plasma. Astrocytes form a cellular syncytium, connected through gap junctions (Fróes & de Carvalho, 1998; Scemes & Giaume, 2006; Giaume, 2010). Ca^{2+}-elevations can be distributed throughout this network by diffusion of IP3/Ca^{2+} through gap junctions as well as ATP-release and sequent activation of P2Y purinergic receptors (Cornell-Bell *et al.*, 1990; Guthrie *et al.*, 1999; Kuga *et al.*, 2011). *In vivo*, astrocytic Ca^{2+} signals occur spontaneously (Hirase *et al.*, 2004; Takata *et al.*, 2011), and in response to neuronal activation (Schummers *et al.*, 2008; Kuga *et al.*, 2011). Downstream of these Ca^{2+} elevations, it has been shown that astrocytes can release neuroactive compounds in a Ca^{2+}-dependent manner. This concept where astrocytes contribute to the signal processing together with neurons has been termed *tripartite synapse* (Araque *et al.*, 1999). It suggests that the functional synapse does not only consist of the pre-and

postsynaptic elements but as well of the astrocytic process and that this three cellular compartments influence each other reciprocally.

In acute slices of different brain areas, neuronal slow inward currents (SICs) due to the activation of NMDA receptors (NMDARs) and slow outward currents (SOCs) due to the activation of GABA-A receptors (GABA-Rs) can be detected in response to glutamate and GABA release by astrocytes (Angulo *et al.*, 2008; Perea *et al.*, 2009; Perea & Araque, 2010). A recent study has indicated that both SICs and SOCs can be recorded in single hippocampal neurons (Le Meur *et al.*, 2012) as well the rapid and dynamic activity of astrocytes correlated with behavior and cognitive functions (Schummers *et al.*, 2008). However the mechanisms of gliotransmitter release and the molecules being released remain unclear, and a clear role of Ca^{2+}-dependent gliotransmission in patho- and physiological conditions is lacking, so that the overall impression left by the literature is that of an intense controversy (Fiacco *et al.*, 2009a; Hamilton & Attwell, 2010; Nedergaard *et al.*, 2010). Moreover, some specific questions still remain not answered, for instance: Can different gliotransmitters be released by the same astrocyte? Are glutamate and/or GABA released in response to the same stimuli? Does the same astrocytic stimulation trigger not only SICs but also SOCs?

One problem for the investigation of specific functions of astrocytes in a tissular context is the lack of a method to selectively stimulate astrocytes (Fiacco & McCarthy, 2004; Crowe *et al.*, 2010). If neurons and astrocytes share the same receptors and transmitter molecules it is difficult to distinguish between a direct effect on neuronal signal transmission and an effect on neurons caused by the activation of astrocytes and subsequent gliotransmission sensed by the neuron. For example, it had been recently proposed that, in the hippocampus, excitatory feedback inhibition is mediated by neuronal adenosine release and not astrocytic ATP release (Lovatt *et al.*, 2012). Methods that have been used for specific activation are mechanical and electrical stimulation, as well as uncaging of Ca^{2+} and IP_3 in astrocytes (Fiacco & McCarthy, 2004; Crowe *et al.*, 2010). These methods have the disadvantage that they cannot be used repetitively or lead to damage of the cell. Also, most studies in acute brain slices and *in vivo* have been based on bulk-loaded membrane-permeable chemical Ca^{2+} organic indicators dyes or with patch-mediated dialysis of Ca^{2+} indicator dyes (Russell, 2011). However, neither of these methods reports on entire astrocytes (Reeves *et al.*, 2011), and

their use has resulted in somewhat inconclusive data on the importance of somatic astrocyte Ca^{2+} signals (Agulhon *et al.*, 2008; Halassa & Haydon, 2010; Hamilton & Attwell, 2010). In the last years, new methods were developed to specifically activate astrocytes (Pascual *et al.*, 2005; Petravicz *et al.*, 2008). Among them, one interesting approach is optogenetics. Light-gated channels are now routinely used to activate neurons since the first introduction of channelrhodopsin-2 (ChR2) in mammalian neurons (Nagel *et al.*, 2003; Boyden *et al.*, 2005). This light-gated channel has also been used in two studies to elevate astrocytic Ca^{2+} in situ (Gradinaru *et al.*, 2009; Gourine *et al.*, 2010; Figueiredo *et al.*, 2011). However, studies have showed that, due to its low Ca^{2+} permeability and fast inactivation, ChR2 cannot evoke consistent Ca^{2+} elevations in astrocytes, which, unlike neurons, lack the voltage-gated Ca^{2+} channels (Li *et al.*, 2012b). In contrary, the light-gated, modified glutamate receptor (LiGluR) (Volgraf *et al.*, 2006; Li *et al.*, 2012a) and Ca^{2+}-translocating variant of channelrhodopsin (CatCh) (Kleinlogel *et al.*, 2011) have emerged as interesting alternatives that could mediate direct Ca^{2+} influx through the plasma membrane and as well been used in astrocytes.
The aim of this work was to evaluate optogenetic tools to manipulate the activity of astrocytes in hippocampal slices and to study the consequences of these specific manipulations on the generation of SICs and SOCs in neurons recorded by means of the patch clamp technique. As a first crucial step, we have tested the specific expression of light-gated channel CatCh in astrocytes by using adeno-associated virus approach (AAV) as well as setting up a system for illumination. In this work, we present some data of viral protein expression analyzed by immunohistochemistry. Here we discussed the limitations on specificity of the virus and the presence of reactive gliosis, which raise a discussion of hope and pitfalls of this methodology and the importance of this study.

Methods

1. *Breeding and genotyping of GLAST creER^{T2} and Cx30 creER^{T2} mice*.

According with pre-established routine of the lab. Genotyping was performed by RT-PCR technique.

2. Surgery and in vivo microinjection of adeno-associated virus subtype 5 (AAV5).

Stereotaxic injection of viral vectors were performed in heterozygous GLAST creER$^{T2+/-}$ (Mori *et al.*, 2006) and Cx30 creER$^{T2\ +/-}$ (Slezak *et al.*, 2007) male and/or female C57BL/6 mice at postnatal day 21-25 (P21-25; 10-17g). Animals were anesthetized by intraperitoneal (i.p) injection of ketamine (52-120mg/kg) and xylazine (4.7-10.7mg/kg) after induction with isoflurane (induction at 5%, maintenance at 1-2.5% vol/vol). After induction of anesthesia, the mice were fitted into a stereotaxic frame (Ultra 963 model), covered with one layer of "space blanket", and shaved. After cleaned the surgical incision site with 70% ethanol, lidocaine (2%) was injected subcutaneously, and the scalp was incised.

Periosteum was gently removed from the part of the inter-parietal bone where a small (<1mm) craniotomy was made (0.5mm mediolateral, -6.2mm anteroposterior, from Bregma) by drilling the bone down to the second tablet (Fine Science Tools-FST, spherical bit, 0.5mm diameter). The remaining bone was cut with a 27 gauge needle. It was made a small incision dura-máter before placing the injection cannula (36 gauge with 45° bevel, Coopers Needle Works Ltd.), so that the upper edge of the bevel was at the parietal cortex surface. The cannula was then lowered 100µm inside the cortex and was left there for 2 minutes, before the infusion. The volume of 1 to 2µL of the vector suspension was infused at 0.1µL/min by a syringe pump (Harvard apparatus model 702208). After 25 minutes, the cannula was removed, lidocaine applied on the wound, and the scalp sutured with single external 5-0 nylon sutures. Animals were given 0.5mL of warm saline subcutaneously, 0.05mL of Antisedan (0.2mg/kg) i.p. and placed under a heating lamp until recovery.

The following adeno-associated virus serotype 5 (AAV5) vectors were used: AAV5.CAG-EGFP (mostly used as control), enconding flexed green fluorescent protein (GFP) reporter, and AAV5.CAG-CatCh.T2A.EYFP, which was encoding flexed yellow flourescent protein (YFP) reporter linked by 2A-peptide sequence with plasmatic membrane photosensible channel, Ca^{2+} translocating

channelrhodopsin – CatCh (Kleinlogel *et al.*, 2011). The 2A-peptide system has been used successfully to generate multiple proteins from a single promoter in many applications (Trichas *et al.*, 2008) and to help mediating the co-expression of a reporter gene with the target cDNA. These virus were purchased at PennVector (University of Pennsylvania School of Medicine, Vector Core).

3. The cre-lox-system and tamoxifen injection protocol

In order to obtain the proper expression of the photoswitchable channel and fluorescent proteins, we used the cre-lox-system which combines cre mouse lines with cre-dependent flexed viral vectors (Kuhlman & Huang, 2008), containing the desired construct under a ubiquitous promoter. The vector contains the construct in inverse transcriptional sense and surrounded by flexed (two times floxed) sides (Atasoy *et al.*, 2008). The cre-recombinase activity on the flexed sides turns the construct in the right sense and therefore activates the construct. Injection of the flexed vector in a mouse expressing the cre-recombinase under a cell specific promoter would lead to a cell specific expression of the construct, which is controlled by a strong ubiquitous promoter.

In order to induce cre-recombinase activity, tamoxifen protocol was performed 24h after the viral stereotaxic injection. At the beginning, this protocol was altered from 5 days of tamoxifen i.p. injections at dose of 1mg/kg to 1 day of 4-hydroxytamoxifen/oil-emulsion (4-hydroxytamoxifen (4-OHT) from Sigma, stored at 4ºC) i.p. at 2mg/kg. These variations occurred in attempt to improve the security of the protocol and minimize damage. Apparently, we have identified that there were no differences on protein expression between the two protocols.

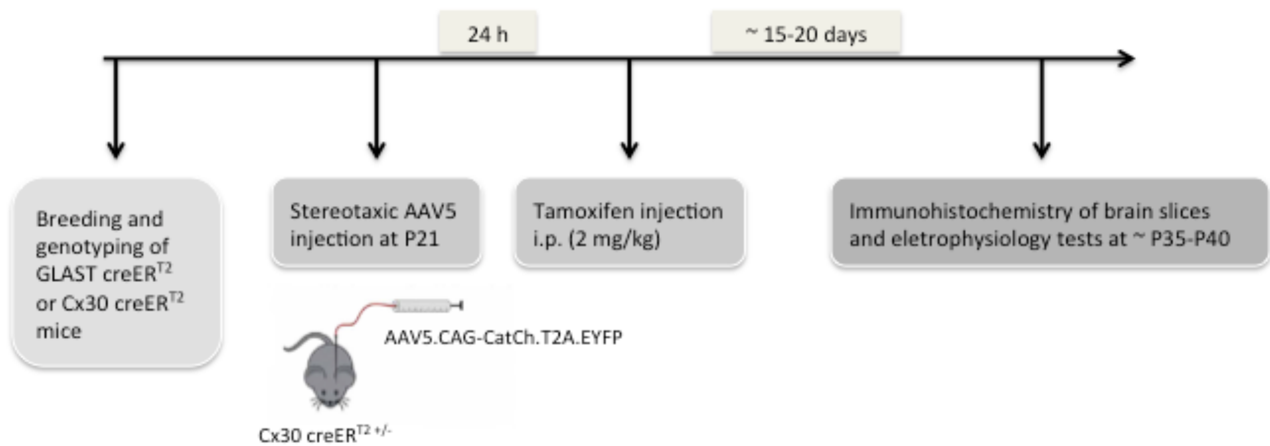
4. Imunohistochemistry

After 15-20 days from tamoxifen injection, mice were deeply anaesthetized and then perfused with cold saline followed by 4% paraformaldehyde (PFA) in phosphate buffered saline and the brains were fixed overnight in PFA. We decided for this period because generally it takes ~ 2 weeks to achieve expression in cells by AAV infection and because it has been suggested that long-term expression (> 3 weeks after AAV injection) can cause toxicity in neurons (Akerboom *et al.*, 2012). Brain sections (60 μm) were cut, incubated with respective antibodies, mounted on glass slides, and coverslipped for imaging on confocal microscopy.

After the specific expression of viral proteins analysis by immunohistochemistry, this project would follow two next steps:

5. Patch clamp recordings in astrocytes hippocampal slices, in order to evaluate the functional expression of viral proteins.

6. And finally, whole cell patch-clamp recordings of neurons in attempt to test whether light stimulation of astrocytes triggers SICs and SOCs.



Viral characterization routine. Before stereotaxic injections**,** the mice were genotyped by RT-PCR. After tamoxifen injection (i.p., 2mg/kg), the mice were conditioned in separate room for at least 15 days to allow the viral protein expression. Immunohistochemistry was performed around postnatal day 35-40 (P35-40) in order to analyse the specific protein expression in astrocytes. Eventually, some eletrophysiology tests were also performed.

Results and discussion

This study was part of a project during my year stage at the Université Paris Descartes in the Laboratory of Neurophysiology and New Microscopies. There are three main points that must be considered here: i) a review of the recent studies on gliotransmission, ii) the major drawbacks of studying astrocyte Ca^{2+} signals, and finally, iii) the hope and pitfalls of a viral characterization approach to target astrocytes.

It's worthy to note that the concept that astrocytes may exert an active and rapid feedback control of the neuronal activity is of great functional importance for understanding the working of the brain under both normal and pathological conditions. However, it is still far from being admitted.

Protoplasmatic astrocytes are electrically silent. However, they may be considered as excitable cells in the sense that they show Ca^{2+} signals, both spontaneously and in response to neuronal activity (Nett *et al.*, 2002; Carmignoto & Fellin, 2006). In spite of the evidence suggesting that Ca^{2+} signals are necessary and sufficient to induce gliotransmitter release, many questions remain, concerning both role and sources of Ca^{2+} signals in astrocytes (Agulhon *et al.*, 2008; Fiacco *et al.*, 2009b; Parpura & Verkhratsky, 2012b). One limiting factor to study Ca^{2+} signaling has been methodological. So far, most studies in acute brain slices and *in vivo* have been based on bulk-loaded membrane-permeable chemical Ca^{2+} organic indicators (Paredes *et al.*, 2008). The membrane-permeable acetoxymethyl (AM) esters, Fluo4-AM and Oregon Green BAPTA1-AM (OGB1-AM), the most popular dyes used to image Ca^{2+} activity in populations of astrocytes, allow imaging the somatic region and the larger proximal processes but leave the very thin distant processes that participate to the tripartite synapse unsampled (Reeves *et al.*, 2011). Additionally, patch-mediated loading of dyes is known to dialyze and disrupt astrocytes functions (Nett *et al.*, 2002) or alters cell physiology (Rand *et al.*, 1994). Another limiting factor has been the lack of specificity of these membrane-permeable Ca^{2+} indicators, which label both neurons and astrocytes (Garaschuk *et al.*, 2006) with cell-type preferences, depending on the indicator, the protocol of application, the brain region, and the age of the animal (Kafitz *et al.*, 2008; Schnell *et al.*, 2012).

In this context, new genetically targeted optical and pharmacological tools have been developed and a number of studies have been showing great advances on the systematic study of Ca^{2+} signals within astrocytes. Nevertheless, and despite of these new tools overcome some limitations of existing methods, they need to be constantly tested. Indeed, it is important to considerer some of the eventual pitfalls of these new approaches and bring them to discussion in order to ensure their reproducible factor.

Here, we showed some data of the viral characterization approach to target astrocytes trough the cre-lox-system with cre-dependent floxed viral vectors (Kuhlman & Huang, 2008). Our major concern was ensuring a specific astrocytic labeling. According with this protocol, specificity should be more stable as it depends on cre-expression within the mouse line. The combination of cell specificity and high expression rate is especially important for high expression levels the light-gated channels. On the other hand, as it is a switch system, even a weak background expression in a cell might be able to switch on a strong construct expression.

The AAV injection protocol for specific expression of proteins in astrocytes is one of the most widely used for astrocyte infection, specially, the AAV serotype 5, with a high tropism for astrocytes (Ortinski *et al.*, 2010). In this study, this approach allowed us to see labeled astrocytes, however we found two main limitations: the lack of specificity and a significant reactive gliosis.

First, as mentioned in methods, we tested the expression of AAV serotype 5 flexed EGFP (green fluorescent protein) under CAG promoter (AAV5.CAG-EGFP) into the hippocampus at P21 of astrocyte specific transgenic mice (GLASTcreER^{T2}) (Slezak *et al.*, 2007), expressing a tamoxifen inducible enzyme cre-recombinase under the GLAST (glutamate aspartate transporter) promoter. Tamoxifen injection (i.p) allowed the cre-recombinase induction (Slezak *et al.*, 2007; de Melo *et al.*, 2012), and it was expected to see the EGFP expression in astrocytes. A relatively large number of cells expressing the viral protein, among them, typical astrocyte morphology, was confirmed by co-localization EGFP/S100β (astrocyte marker) and recognized at CA1 region **(Suplementar 1)**. Surprisingly, at the dentate gyrus we observed the expression of the virus in some neurons by the co-localization of EGFP with the neuronal marker, NeuN **(Figure 1)**. It has known that GLAST protein is expressed in the Radial Glia (Shibata *et al.*, 1997), and thus, in the newly

neurons formed at the dentate gyrus, which is an area of adult neurogenesis (Namba *et al.*, 2005; Ming & Song, 2011). However, we did not expect that by injecting at the CA1 region, the virus was able to spread until the dentate gyrus, which was the case. To investigate the non-specific expression of viral proteins through the adult neurogenesis hypothesis, transgenic approaches were performed by crossing GLASTcreER^{T2} mouse line with floxed GCaMP3 mouse line (Zariwala *et al.*, 2012) and an immunohistochemistry labeling with doublecortin, which allow us recognize immature or newly neurons, were performed into dentate gyrus. The results confirm the co-localization EGFP/doublecourtin in some newly neurons and corroborates with the neurogenesis studies **(Suplementar 2)**.

Following these results, we decided to use another lineage of transgenic mice expressing cre-recombinase under control of astrocytic Cx30 (connexin 30) promoter (Cx30creER^{T2}) (Slezak *et al.*, 2007). At this time, we tested the expression of the virus construct, AAV5 econding YFP (yellow fluorescent protein) and the Ca^{2+}-translocating variant of channelrhodopsin (CAG-CatCh.T2A.EYFP) at P21 into hippocampus of these animals. We recover the co-localization YFP/S100B at CA1, indicating the well expression of the virus in astrocytes **(Figure 2)**. However, we also found a great number of labeled neurons at the dentate gyrus by the co-localization YFP/NeuN. Indeed, the Cx30creER^{T2} lineage has not been well characterized as GLASTcreER^{T2} lineage (Mori *et al.*, 2006), but genetics crosses between the transgenic mice Cx30creER^{T2} and floxed GCaMP3 mouse line showed no neuronal expression of the reporter **(Suplementar 3)** (Wallraff *et al.*, 2006; Fiacco *et al.*, 2009b), which brought us some hope at the beginning. However, these results confirm other studies which suggested that Cx30 protein could be expressed in Radial Glia and, therefore, in newly neurons as well (Kunze *et al.*, 2009). An additional alternative strategy to enhance the specification into the hippocampus could be to silence the construct expression in neurons by adding a miRNA targeting site directly after the construct (Brown *et al.*, 2006). Binding of miRNA on the transcribed mRNA prevents construct translation. The miRNA124 targeting site has already been used in lentiviral and AAV vectors to prevent expression in neurons, but it showed a viral titer dependent efficiency (Colin *et al.*, 2009; Karali *et al.*, 2011).

Instead of that and following these observations, we decided to move the injections from hippocampus to cortex of Cx30 creER^{T2} mouse line, in attempt to prevent this neuronal labeling.

Once more, we found a good astrocytic expression, confirmed by co-localization of EGFP with S100B and only a few neurons were labeled **(Figure 3)**. Comparatively with the hippocampus, the number of neurons expressing EGFP protein in the cortex was lower, but this neuronal expression was not explained by neurogenesis.

On the other hand, inflammation may change the transcriptional activity of the cells. For this reason and to investigate whether the viral injection could activate inflammatory process, we performed a control for astrogliosis through GFAP (glial fibrilar acidic protein) expression, and microglial Iba1 expression. A few days after the viral injection, layer 5 of the ipsilateral cortex injection showed that there was indeed an enhanced of GFAP and Iba1 expression than the contralateral cortex injection, suggesting reactive gliosis **(Figure 4)**. This was an important limitation of the viral delivery strategies, and needed to be taken into account. Other studies have already observed a significant dose-dependent reactive gliosis after intracerebral injection of AAV (Reimsnider *et al.*, 2007; Ortinski *et al.*, 2010). Since gliosis is associated with changes of several signaling pathways in astrocytes (Hamby *et al.*, 2012), it will be important to develop alternative approaches to study the role of astrocytes in physiological conditions. Introducing a sequence encondig the VIVIT peptide that interferes with the calcineurin/nuclear factor of activated T-cells signaling pathway and down regulates GFAP overexpression (Furman *et al.*, 2012), may reduce AAV-induced gliosis. Another alternative could be replacing viral constructs by transgenic floxed/tetO mouse lines. Several floxed (Slezak *et al.*, 2012; Zariwala *et al.*, 2012) and tetO (Fiacco *et al.*, 2007; Agulhon *et al.*, 2010) mouse lines of interest have been generated. Despite of consuming some time, this strategy is advantageous since it does not require surgery for viral injections (Li *et al.*, 2013).

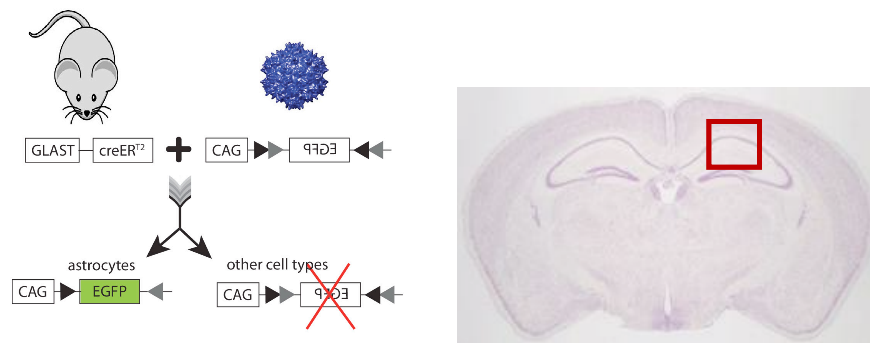
Overall, this study brought us some interesting findings about the viral injections approaches and calls the attention for the limitations of this methodology in the astrocyte field. The need for controls will continue to be important especially because astrocytes in different parts of the brain are heterogeneous (Zhang & Barres, 2010) and display age-dependent changes in function (Sun *et al.*, 2013).

Uptades so far and perspectives:

Meanwhile, the research group at Université Paris-Descartes has been trying to solve some of the limitations presented in this work by testing new approaches, such as:

1. Viral injections in Cx30 creER^{T2} pups, at postnatal day 3 (P3), in order to reduce reactive gliosis for the virus, after induction of cre expression later (Gholizadeh *et al.*, 2013).
2. Also, they are testing viral injections with glass pipette instead of metal needle.
3. Transgenic approach by crossing Cx30 creER^{T2} with floxed ChR2 mouse line.

Figures



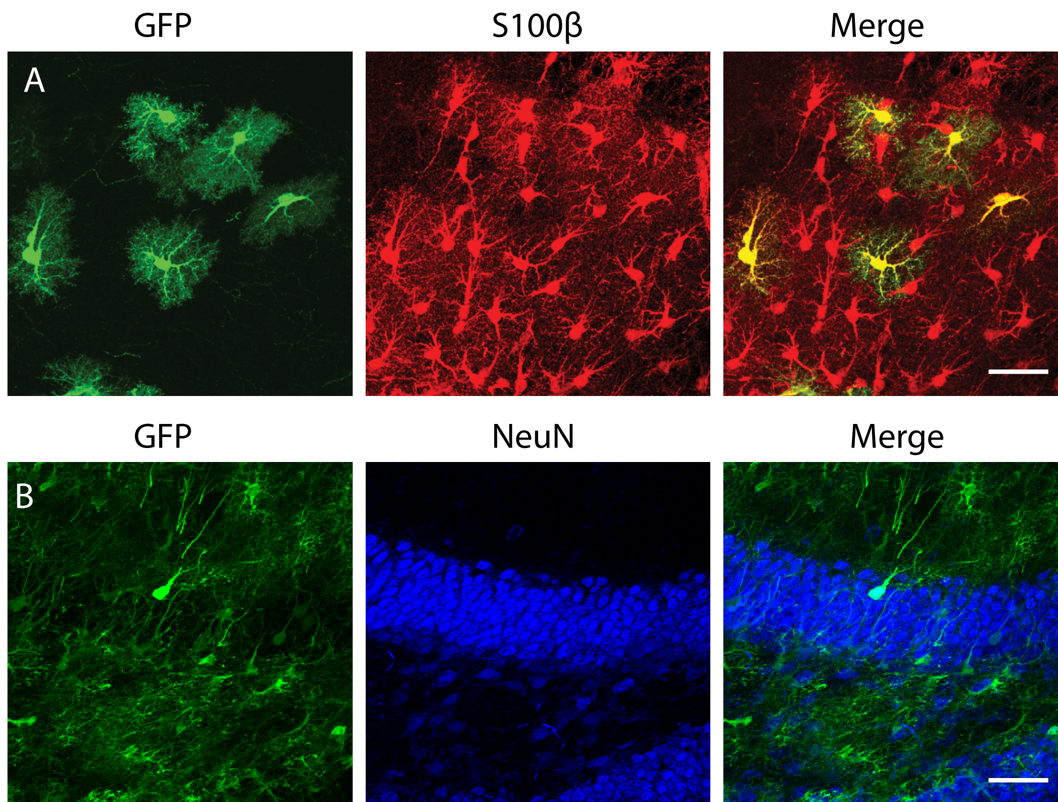
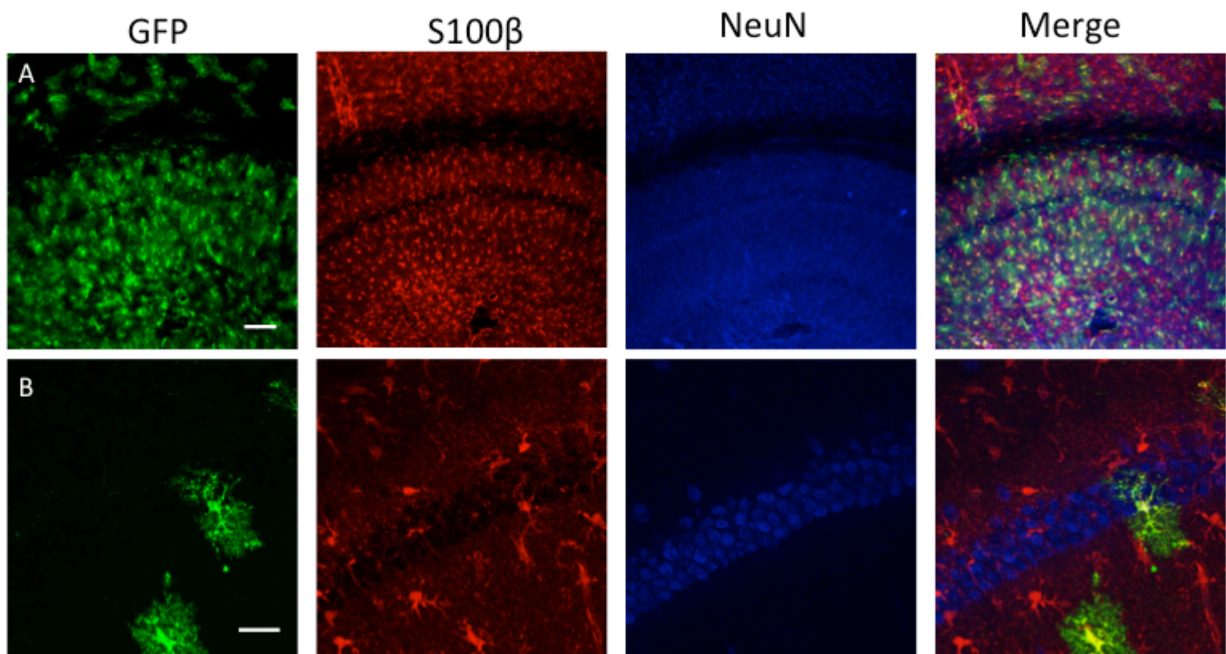


Figure 1. Characterization of EGFP expression in GLAST creER$^{T2+/-}$ mouse line. AAV5.CAG.FLEX.EGFP.WPRE.bGH virus was injected in P21 GLAST creER$^{T2+/-}$ mouse hippocampus and analyzed by immunohistochemistry at least two weeks after. As activity of GLAST promoter is essential for activation of the flexed construct, EGFP expression is just expected in astrocytes and not in other cell types and should occur after induction with tamoxifen (i.p., one day after the stereotaxic injection of the virus). The pictures are confocal stack images of the CA1 and dentate gyrus regions. (A) In CA1, most of the transfected cells were astrocytes whereas (B) in dentate gyrus some neurons are expressing EGFP in great density. Scale bars 50 µm in A and 70 µm in B.



Suplementar 1. Characterization of EGFP expression in GLASTcreER$^{T2+/-}$ mouse line. AAV5.CAG.FLEX.EGFP.WPRE.bGH virus was injected in P21 GLASTcreER$^{T2+/-}$ mouse hippocampus and analyzed by immunohistochemistry at least two weeks after. The expression was induced by tamoxifen i.p. injection one day after the stereotaxic injection of the virus. The pictures are confocal stack images of the CA1 region. Scale bars 100 μm in A and 50 μm in B.

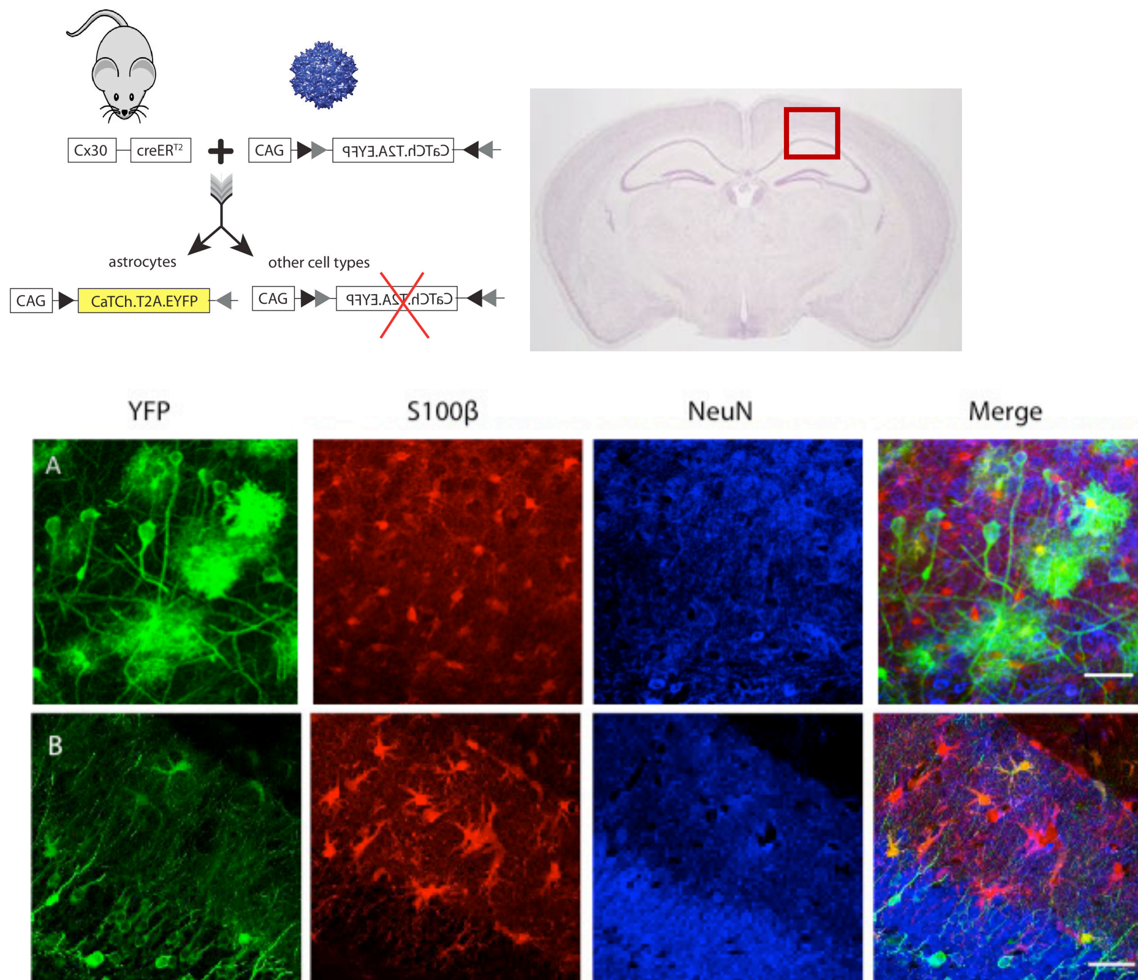
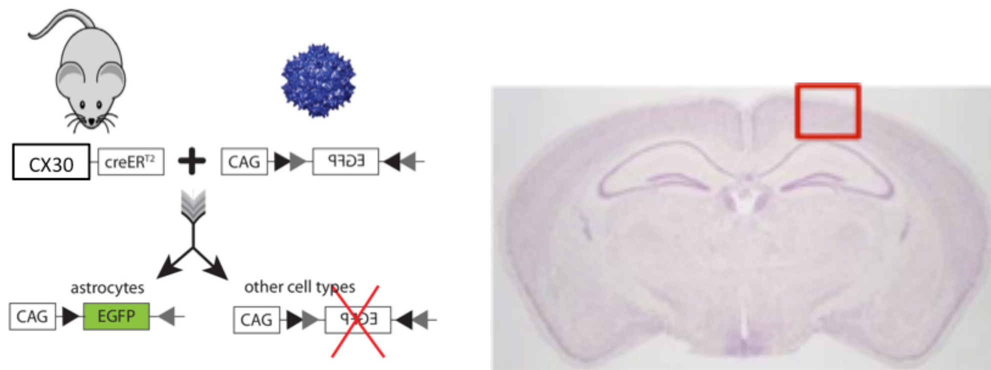


Figure 2. Characterization of EYFP expression in Cx30creER$^{T2+/-}$ mouse line. AAV5.CAG.FLEX.CaTCh.T2A.EYFP.WPRE.bGH virus was injected in P21 Cx30 creER$^{T2+/-}$ mouse hippocampus and analyzed by immunohistochemistry at least two weeks after. As activity of Cx30 promoter is essential for activation of the flexed construct, EYFP expression is just expected in astrocytes and not in other cell types and should occur after induction with tamoxifen (i.p., one day after the stereotaxic injection of the virus). The pictures are confocal stack images of the CA1 and dentate gyrus regions. (A) In CA1, most of the transfected cells were astrocytes whereas (B) in dentate gyrus some neurons are expressing EGFP in great density. Scale bars 50 µm in A and B.



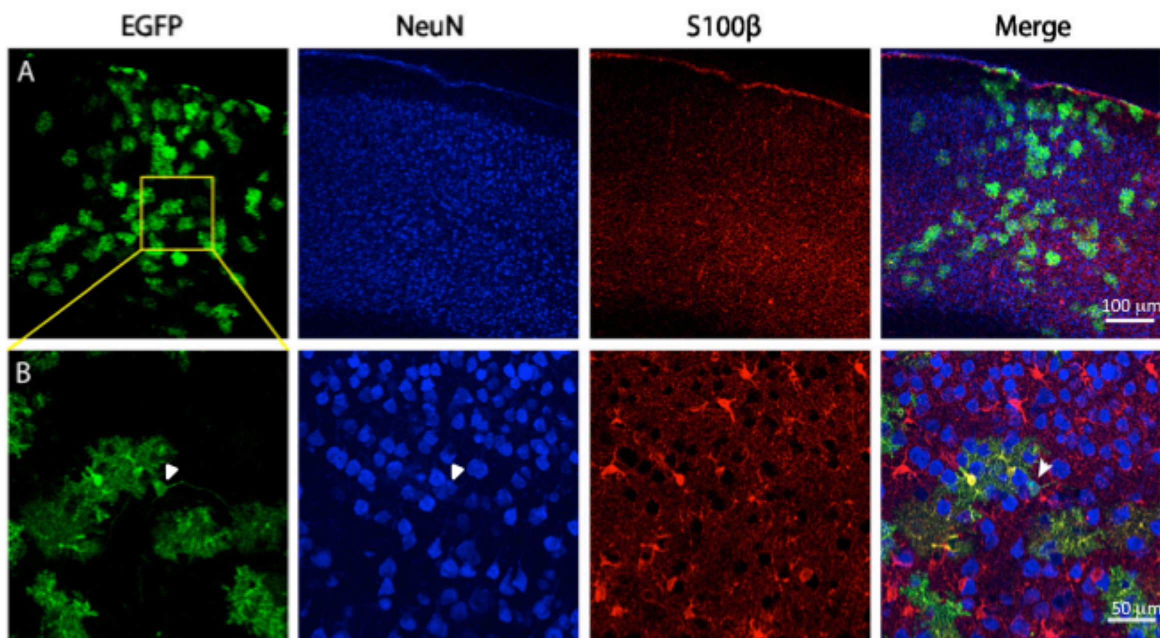


Figure 3. Characterization of EGFP expression in Cx30creER$^{T2+/-}$ mouse line. AAV5.CAG.FLEX.EGFP.WPRE.bGH virus was injected in P21 Cx30 creER$^{T2+/-}$ mouse cortex and analyzed by immunohistochemistry at least two weeks after. As activity of Cx30 promoter is essential for activation of the flexed construct, EGFP expression is just expected in astrocytes and not in other cell types and should occur after induction with tamoxifen (i.p., one day after the stereotaxic injection of the virus). The pictures are confocal stack images of the neocortex.

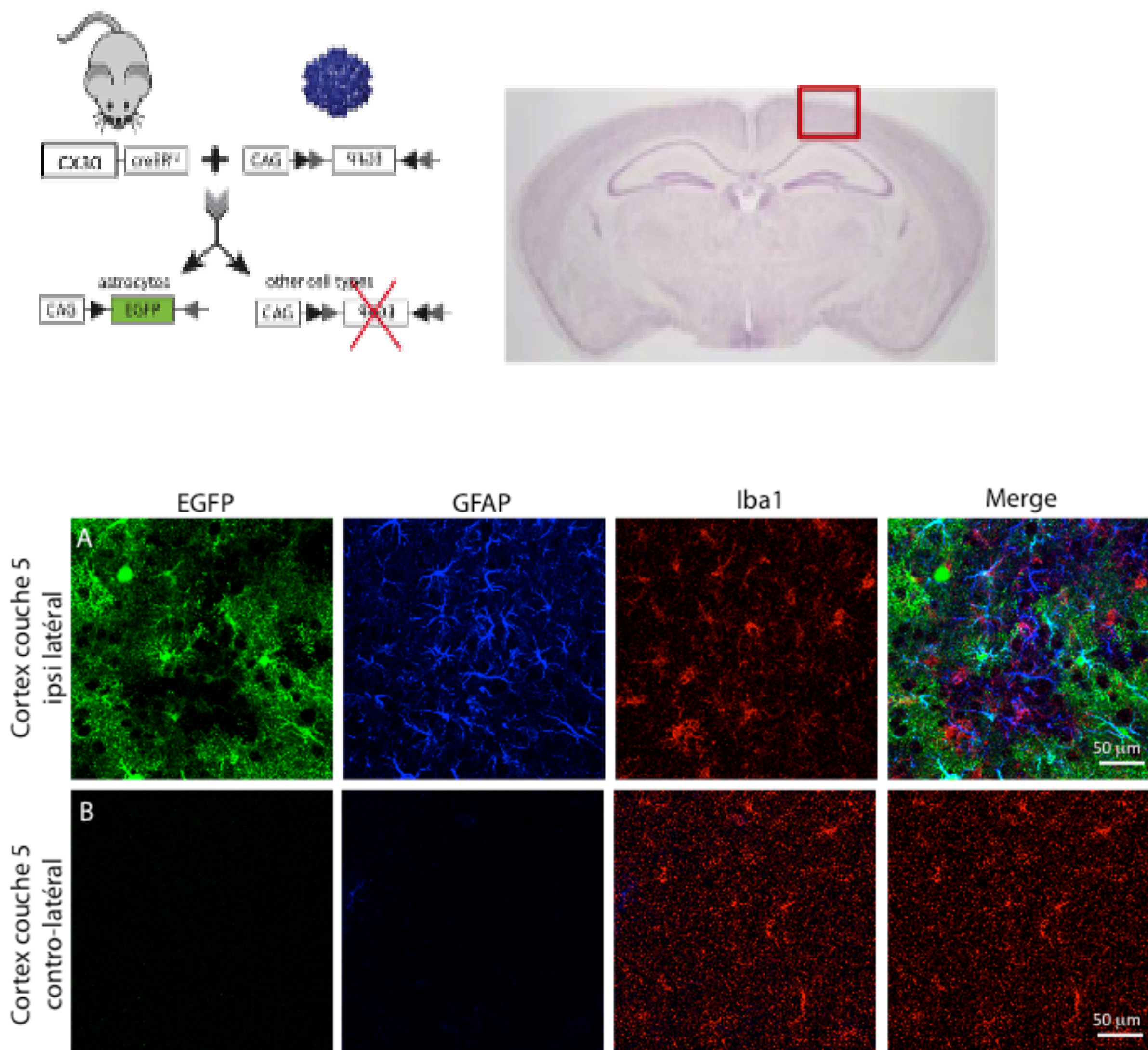
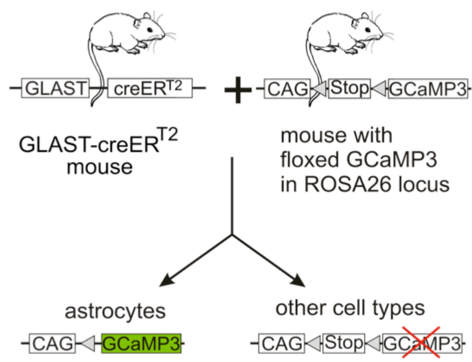
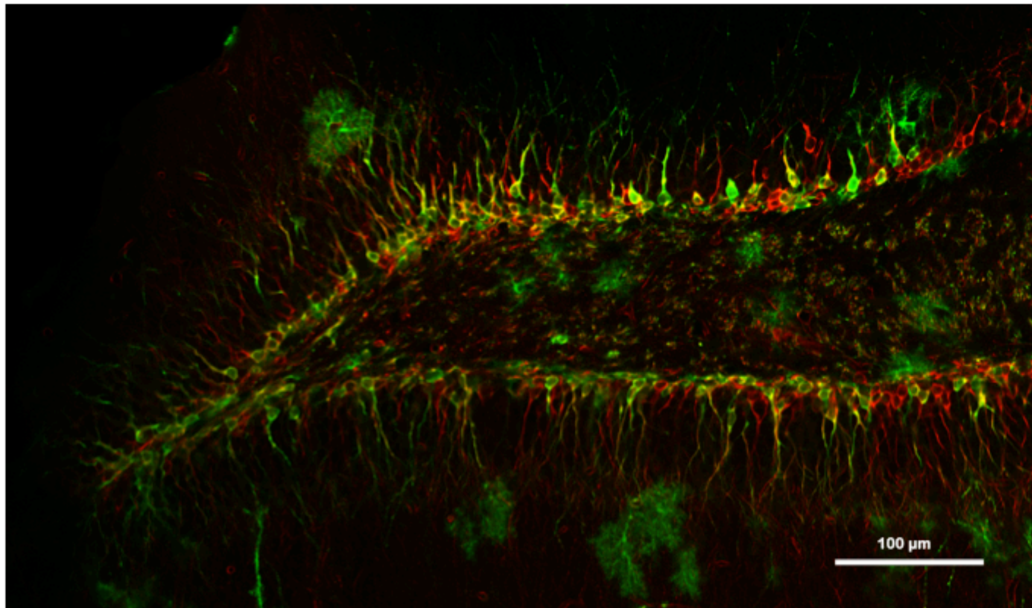
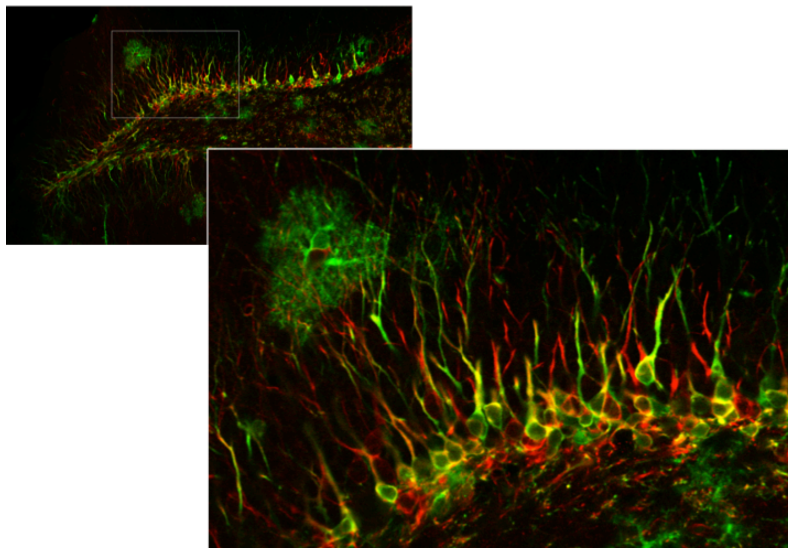


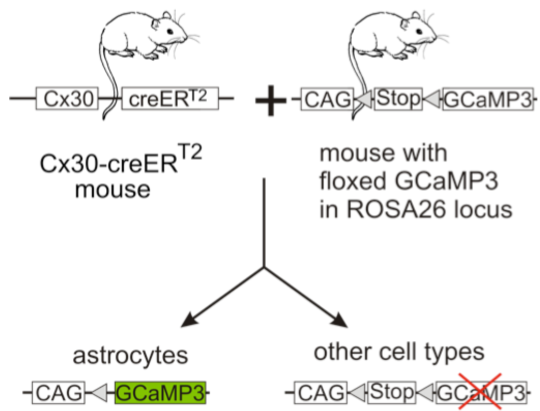
Figure 4. Characterization of EGFP expression in Cx30creER$^{T2+/-}$ mouse line. AAV5.CAG.FLEX.EGFP.WPRE.bGH virus was injected in P21 Cx30 creER$^{T2+/-}$ mouse cortex and analyzed by immunohistochemistry at least two weeks after. The pictures are confocal stack images of the neocortex (layer 5) and the strong labeling of GFAP and Iba1 ipsi-lateral to the injection indicates reactive gliosis.

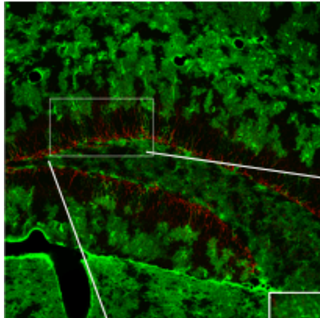


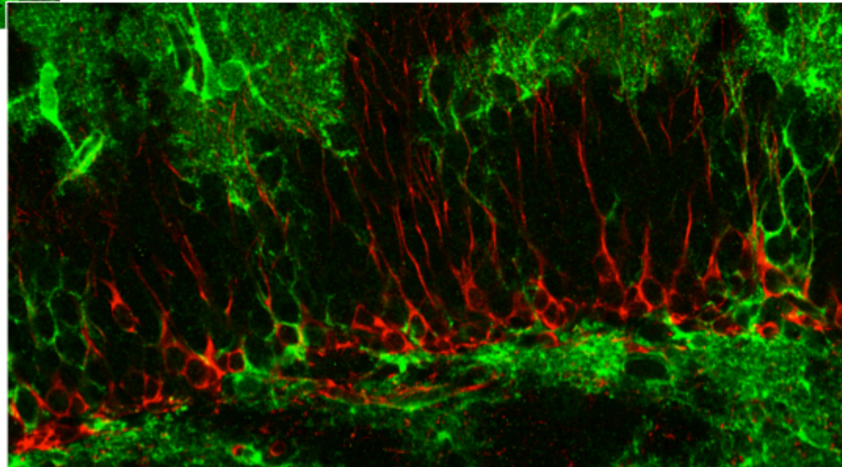




Suplementar 2. Investigation of non-specific expression of astrocytic proteins in dentate gyrus by transgenic approach which combines GLASTcreER^{T2} mouse line with floxed GCaMP3 mouse line. EGFP expression (green) and doublecortin labeling (red). These results indicate the co-localization EGFP/doublecourtin (yellow) in some newly neurons.







Suplementar 3. Investigation of non-specific expression of astrocytic proteins in dentate gyrus by transgenic approach which combines Cx30creER^{T2} mouse line with floxed GCaMP3 mouse line. EGFP expression (green) and doublecortin labeling (red).

References

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PARTE III

3. Discussão

De acordo com os capítulos 1 e 2 desta tese foi possível aprofundar os estudos sobre a importância das células astrogliais, reforçando o conceito de que astrócitos não podem mais ser caracterizados apenas como células passivas mas como também células ativas do SNC.

Anticolinesterásicos ainda são alternativa terapêutica mais utilizada para estabilizar a atividade colinérgica neuronal e, assim, amenizar a disfunção cognitiva de pacientes com DA ou outros tipos de demências (Ahmed *et al.*, 2013; El-Malah *et al.*, 2014), entretanto seus efeitos nas células astrogliais são ainda muito pouco estudados. No primeiro capítulo, o objetivo foi avaliar se a modulação do sistema colinérgico através da inibição da AChE em culturas primárias de astrócitos era capaz de alterar a secreção de S100B, uma proteína predominantemente expressa em astrócitos, e que já teve suas funções apontadas como relevantes para o entendimento dos processos neurodegenerativos da DA em um grande número de estudos (Peskind *et al.*, 2001; Wilcock & Griffin, 2013). Dentre os IAChE testados, apenas HupA foi capaz de aumentar a secreção da S100B neste modelo, enquanto que tacrina não teve esse efeito agudo e apenas reduziu o conteúdo intracelular de S100B em 24 h. Estes efeitos poderiam ser explicados pela evidente diferença entre os compostos (Mehta *et al.*, 2012), entretanto seus potencias inibitórios sobre a enzima AChE *in vitro* são muito semelhantes (Zhao & Tang, 2002). Nesse sentido, é importante ressaltar que IAChE também podem apresentar outras atividades moduladoras, especialmente neuroprotetoras diante de modelos de dano (Racchi *et al.*, 2004; Sugimoto, 2008), que não estão necessariamente relacionadas com a inibição da enzima AChE (Pepeu & Giovannini, 2009). Este conceito de “droga com múltiplos alvos” tem sido bastante relacionado com as diferentes funções da HupA,

em particular (Zhang & Tang, 2000; Peng *et al.*, 2007; Zhang *et al.*, 2008), acumulando mais evidências terapêuticas deste novo IAChE, embora ainda não aprovado pelo *Food and Drug Administration* (FDA) (He *et al.*, 2007; Wang *et al.*, 2009a; Xing *et al.*, 2014).

Os resultados da secreção de S100B através do tratamento com IAChE motivaram a investigação da segunda parte deste capítulo: cabia verificar se os receptores colinérgicos também estavam envolvidos na secreção de S100B. Dessa vez, as culturas de astrócitos foram tratadas com agonistas (nicotina e carbacol) e antagonistas (mecamilanima e escopolamina) colinérgicos nicotínicos e muscarínicos, respectivamente. Mostramos pela primeira vez que a nicotina foi capaz de aumentar a secreção de S100B *in vitro*, e que este efeito foi bloqueado pelo seu antagonista nicotínico, mecamilamina, sugerindo que em condições basais a ativação dos receptores nicotínicos nos astrócitos é um fenômeno funcional e relevante para esta atividade astroglial. Receptores nicotínicos são canais iônicos ativados por ligantes e podem ser expressos em diversos tipos celulares não-neuronais (Sharma & Vijayaraghavan, 2002; Wessler & Kirkpatrick, 2008). Alguns estudos já mostraram a expressão funcional em culturas de astrócitos (Xiu *et al.*, 2005; Shen & Yakel, 2012; Wang *et al.*, 2013). De um modo geral, estes receptores exibem alta permeabilidade ao Ca^{2+}, por isso sua ativação resulta em um aumento nos níveis de Ca^{2+} intracelular (Sharma & Vijayaraghavan, 2001; Oikawa *et al.*, 2005b; Grybko *et al.*, 2010), que por sua vez pode modular diversas funções (Hernández-Morales & García-Colunga, 2009).

Outros estudos no nosso grupo já evidenciaram o papel de diferentes sistemas de neurotransmissão na modulação da secreção de S100B, através do uso de fármacos conhecidos, geralmente envolvidos na terapêutica de uma desordem neuronal específica (Tramontina *et al.*, 2008b; Nardin *et al.*, 2011; Herrmann *et al.*,

2012). Além disso, um outro estudo reportou o potencial secretagogo do solvente orgânico DMSO dependente da mobilização de Ca^{2+} do retículo endoplasmático *in vitro*, além de também ter caracterizado o perfil de diferentes tipos de respostas celulares ao Ca^{2+} (Leite *et al.*, 2010 não publicado). Apesar do mecanismo de ação não estar definido, o acúmulo destes estudos sugere a existência um mecanismo comum: o aumento dos níveis de Ca^{2+} intracelular – um processo consequente ao tratamento com agonista nicotínico, por exemplo, que caracterizaria um tipo de ativação astrocítica *in vitro* e que por sua vez, promoveria a secreção de proteínas tróficas, como a S100B (Davey *et al.*, 2001b; Sakatani *et al.*, 2008), ou outras substâncias neuroativas. Estudos *in vivo* também tentam entender como a proteína é secretada, entretanto tais estudos estão mais focados na alteração dos níveis de S100B como marcador biológico em potencial para diversas doenças (Kleindienst & Ross Bullock, 2006; de Oliveira *et al.*, 2008; Steiner *et al.*, 2009; Schroeter *et al.*, 2013).

É válido notar que a investigação da proteína S100B em estudos comportamentais foi uma das primeiras evidências da participação dos astrócitos na modulação da plasticidade sináptica (Janus *et al.*, 1995; Nishiyama *et al.*, 2002). O exato mecanismo de ação da S100B sobre este processo ainda não está totalmente esclarecido. De qualquer modo, o fato da S100B ser secretada e promover aumento nos níveis de Ca^{2+} tanto em neurônios como em astrócitos (Barger & Van Eldik, 1992), bem como também, melhorar oscilações gama induzidas por kainato (Sakatani *et al.*, 2008), sugere que esta proteína possui um papel relevante nos processos fisiológicos cerebrais de aprendizado e memória. Obviamente que esta interpretação deve também incluir as possíveis alterações da cobertura sináptica astrocítica promovidas pelo efeito da S100B sobre o citoesqueleto (Selinfreund *et al.*, 1990).

O segundo capítulo apresentou os resultados preliminares da primeira parte de um estudo sobre o impacto da ativação da sinalização de Ca^{2+} nos astrócitos e sua influência na atividade neuronal. Consideradas formas primárias de excitabilidade astrocítica, as elevações de Ca^{2+} intracelular (Pivneva *et al.*, 2008) parecem modular significativamente a atividade do encéfalo: durante o desenvolvimento através do crescimento de neuritos (Kanemaru *et al.*, 2007), diante da transmissão sináptica neuronal (Angulo *et al.*, 2004; Fellin *et al.*, 2004; Kozlov *et al.*, 2006; Nestor *et al.*, 2007), durante potenciação de longa duração (Fellin *et al.*, 2006; Jourdain *et al.*, 2007; Perea & Araque, 2007b), depressão heterossináptica (Andersson *et al.*, 2007; Chen *et al.*, 2013), probabilidade de liberação pré-sináptica (Fiacco & McCarthy, 2004) e acoplamento do fluxo cerebral sanguíneo para neurônios ativos (Iadecola & Nedergaard, 2007). Em todos estes casos, a liberação de transmissores pelos astrócitos dependente de Ca^{2+} é o mecanismo apontado como responsável pelos efeitos mediados pelos astrócitos. Estas evidências sugerem que na ausência das elevações de Ca^{2+}, tanto intrinsicamente como também em resposta a atividade neuronal, a função cerebral e o comportamento estariam prejudicadas. Entretanto, o conceito de gliotransmissão ainda é muito discutido (Agulhon *et al.*, 2008; Fiacco *et al.*, 2009a). De fato, investigar as funções dos astrócitos *in situ* é muito desafiador e um crescente número de trabalhos concorda que são necessários novos métodos para ativar os astrócitos seletivamente (Hamilton & Attwell, 2010) e avaliar seus efeitos (Shigetomi *et al.*, 2013). Técnicas eletrofisiológicas convencionais estimulam astrócitos e neurônios ao mesmo tempo, inevitavelmente. Estimulação intracelular dos astrócitos diretamente também é complicado, já que a resistência de entrada destas células é baixa e a despolarização gerada pelo eletrodo no soma não consegue se propagar para as complexas estruturas gliais. Neste estudo, utilizamos a optogenética (Fenno *et al.*, 2011; Tye & Deisseroth, 2012), uma ferramenta que

combina métodos ópticos e biologia molecular e que pode ser usada tanto para monitorar opticamente vários processos nas células de interesse com também controlar sua atividade pelo uso de luz (Figueiredo *et al.*, 2011).

Entretanto, primeiramente, foi necessário testar se o sistema de distribuição desta ferramenta era funcional e reproduzia os efeitos já demonstrados em outros trabalhos. Nesse sentido, testamos o sistema de distribuição viral que consiste na combinação de um constructo viral (contendo as proteínas fotossensíveis de interesse) com camundongos transgênicos adultos que expressavam a proteína cre-recombinase dependente de um promotor celular específico para os astrócitos (Kuhlman & Huang, 2008). Esta parte do trabalho era crucial para a continuidade dos experimentos. Como o objetivo era estudar o efeito da ativação dos astrócitos sobre a transmissão neuronal, mesmo uma ativação mínima, de poucos neurônios, deve ser problemática para interpretar os resultados, visto que a ativação de um único neurônio na vizinhança pode ter um efeito mais expressivo na transmissão sináptica do que ativação de um astrócito. É preciso ressaltar que somente a partir da expressão funcional específica de proteínas fotossensíveis em astrócitos que a investigação poderia continuar.

Dentre os diversos sistemas virais de expressão de proteínas específicos para astrócitos, utilizamos o sistema adenovirus associado (AAV), pois ofereceu as seguintes vantagens: i) uma vez que se tem o constructo viral pronto, pode ser injetado no encéfalo e restringe a expressão em áreas cerebrais de interesse; ii) com o uso de promotores específicos é possível restringir a expressão do plasmídeo por tipo celulares específicos; iii) AAV parece difundir mais facilmente e induz menos astrogliose do que lentivirus; iv) AAV são menos prejudiciais ao experimentador e sua manipulação é simples; v) disponibilidade de muitos AAV no mercado (Penn Vector Core). Esta técnica é mais flexível e mais rápida que a criação de uma nova linhagem

de camundongos. De um modo geral, estes vetores virais existem em diversos sorotipos naturais (AAV 1-9), e todos podem ser usados para transfecção do SNC (Liu *et al.*, 2008; Gholizadeh *et al.*, 2013). AAV5, utilizado em estudos para inserção de constructos em astrócitos (Ortinski *et al.*, 2010; Xie *et al.*, 2010), foi o vetor escolhido. Este vetor continha o constructo com o sentido transcripcional invertido e envolto por dois sítios *flexed* (duas vezes *floxed*) (Atasoy *et al.*, 2008). A atividade da cre-recombinase sobre os sítios *flexed* vira o constructo para o sentido correto e ativa sua transcrição. Para induzir a atividade da cre-recombinase faz-se necessário a administração de tamoxifeno, logo após a injeção intracerebral do vírus. Nesse sistema, o tamoxifeno funciona como ligante sintético dos receptores de estrogênio combinados geneticamente a cre-recombinase, permitindo o controle externo da atividade desta enzima (Feil *et al.*, 1997; Sauer, 1998; Branda & Dymecki, 2004).

Duas linhagens animais para este sistema cre-lox, sob a orientação de diferentes promotores astrocíticos, foram disponibilizadas para este estudo: GLAST-CreER^{T2} (Mori *et al.*, 2006) e Cx30-CreER^{T2} (Slezak *et al.*, 2007). Cada qual foi combinada com dois constructos virais diferentes, tal como o AAV5 codificando a proteína fluorescente verde (EGFP) sob a orientação do promotor CAG (AAV5.CAG-EGFP) e o AAV5 codificando proteína fluorescente amarela (YFP) juntamente com a variação do canal *channelrodopsina* translocador de Ca^{2+} e ativado por luz (AAV5.CAG-CatCh.T2A.EYFP). As possibilidades para combinações entre as linhagens de camundongos e os diferentes constructos virais foram diversas. Os primeiros testes combinaram a linhagem GLAST-CreER^{T2} com AAV5.CAG-EGFP, e a expressão da GFP nas células foi analisada através de ensaios em imunohistoquímica, que utilizou marcadores específicos para astrócitos (S100B ou GFAP) e neurônios (NeuN). Já neste primeiro teste verificamos uma boa expressão viral nos astrócitos, na região de CA1 do hipocampo. Entretanto, alguns neurônios no

giro denteado expressaram GFP indicando uma limitação na especificidade desta metodologia. Isso pode ser explicado pelo fato de que o promotor GLAST ainda pode estar ativo em células progenitoras neuronais presentes no giro denteado, região conhecida pela neurogênese adulta de mamíferos (Ming & Song, 2011; Imayoshi *et al.*, 2012).

Por esse motivo, testou-se outra linhagem com promotor para conexina30, Cx30-CreER^{T2}, agora combinado ao AAV5.CAG-CatCh.T2A.EYFP. Mais uma vez observou-se a expressão dos astrócitos em CA1, mas novamente a expressão inespecífica em alguns neurônios no giro denteado. Dessa vez, não tínhamos um argumento que sustentasse a ativação do promotor de Cx30 nos neurônios desta região, visto que os estudos com esta linhagem ainda não tinham sido bem caracterizados. O grupo realizou outros testes genéticos através do cruzamento entre a linhagem Cx30-CreER^{T2} e a linhagem que expressava GCamP3 (Akerboom *et al.*, 2012) e não havia sido demonstrado expressão neuronal deste promotor (Wallraff *et al.*, 2006; Gosejacob *et al.*, 2011).

A próxima estratégia foi mudar o local de injeção do vírus do hipocampo para o córtex. No início, injetamos o vírus que só expressava a GFP (AAV5.CAG-EGFP) nos animais transgênicos Cx30-CreER^{T2}. Verificamos a expressão da proteína GFP nos astrócitos, mas também nos neurônios, apesar de que dessa vez, em uma menor escala do que foi visto no hipocampo. Este resultado não pôde ser explicado por neurogênese, entretanto, por outro lado, ativação de processos inflamatórios são conhecidos por alterar a atividade de transcrição das células (Rosi *et al.*, 2005; Abbasian *et al.*, 2012). Dias depois da injeção viral, foi possível observar um aumento na marcação para proteínas GFAP e Iba1 ipsilateral a injeção quando comparada ao lado contralateral a injeção, sugerindo gliose reativa. Esta limitação já tinha sido observada em outros estudos (Reimsnider *et al.*, 2007; Ortinski *et al.*, 2010) e

precisou ser levada em consideração. De fato, a gliose reativa está associada com mudanças significativas nas vias de sinalização dos astrócitos (Agulhon *et al.*, 2012; Hamby *et al.*, 2012), e alternativas para se estudar o papel dos astrócitos na transmissão sináptica em condições fisiológicas precisam ser desenvolvidas.

4. Conclusões

De acordo com os resultados obtidos no primeiro estudo, a secreção de S100B foi estimulada pelo tratamento com HupA, diferentemente do tratamento com tacrina, reforçando a ideia de que IAChEs, como a HupA, não atuam exclusivamente sobre a atividade da AChE. Ainda, mostrou-se pela primeira vez que ativação de receptores nicotínicos através do tratamento com nicotina promoveu um aumento na secreção de S100B, o qual foi bloqueado pelo antagonista nicotínico mecamilamina. Tal caracterização corrobora com outros estudos que avaliaram a secreção de S100B dependente da ativação de outros receptores, como glutamatérgicos, dopaminérgicos e serotoninérgicos. O mecanismo exato que descreve a secreção de S100B ainda não está definido, mas especula-se por um mecanismo comum de ativação dos astrócitos representado por alterações nos níveis de Ca^{2+} intracelular.

Com objetivo de entender melhor as consequências agudas da ativação dos astrócitos no funcionamento dos neurônios na região CA1 do hipocampo, utilizamos ferramentas em optogenética. Tal metodologia inclui a expressão de canais de Ca^{2+} sensíveis a luz combinada a animais transgênicos do tipo cre-recombinase induzível, sob o comando de um promotor específico para astrócitos. Primeiramente, era necessário caracterizar a expressão específica destes canais nos astrócitos através de análises por imuno-histoquímica. Os resultados deste segundo estudo mostraram uma boa expressão das proteínas virais em astrócitos em CA1, entretanto,

foi possível também observar a expressão inespecífica em neurônios no giro denteado, região notadamente conhecida pela neurogênese adulta no hipocampo. Após uma série de testes, alteramos a linhagem animal de GLASTcreER^{T2} para Cx30creER^{T2} e movemos a região de estudo do hipocampo para o córtex. Dessa vez, os resultados revelaram um aumento na expressão das proteínas GFAP e Iba1, caracterizando uma significativa gliose reativa no lado ipsi-lateral a injeção viral. Ainda que as metodologias para o entendimento das funções dos astrócitos estejam se aperfeiçoando cada vez mais, estes resultados apontam limitações metodológicas importantes que devem ser discutidas, para que novas soluções possam geradas e testadas.

5. Considerações finais

Nos últimos anos, nosso entendimento sobre os astrócitos e seu modo de se comunicar com as células vizinhas e vasos sanguíneos tem aumentado rapidamente. Em algumas áreas de pesquisa, novos dados, embora ainda incompletos, já parecem bastante esclarecedores e convincentes; em outras áreas, entretanto, ainda estão fragmentados, sugerindo algumas informações que apenas representam a ponta do iceberg.

Funções tróficas ou de sinalização biológica da proteína S100B representam uma, dentre as muitas formas específicas de comunicação dos astrócitos com outras células do SNC. Apesar do grande número de estudos sobre as funções desta proteína, sua relação com os processos cognitivos e memória ainda estão pouco explorados, e se especula se o mecanismo de secreção pode ser explicado como uma consequência da ativação peculiar dos astrócitos através de alterações nos níveis de Ca^{2+}. Evidências que caracterizam o papel de diferentes

sistemas neuronais na estimulação da secreção de S100B, ajudam a entender melhor o papel dos astrócitos no SNC. Apesar de seus efeitos também influenciarem o funcionamento dos astrócitos, IAChE e agonistas colinérgicos eram estudados apenas no sentido de amenizar o prejuízo cognitivo neuronal causado pela doença de Alzheimer. Atualmente, a ideia de que fármacos possam desempenhar “multi-tarefas” é muito bem-vinda, especialmente se podem melhorar o papel trófico dos astrócitos de alguma maneira.

A sinalização pelo Ca^{2+} intracelular também parece desempenhar um papel central na comunicação bidirecional entre astrócitos e neurônios principalmente porque é desencadeada por neurotransmissores liberados durante a atividade sináptica, e que por sua vez vão promover a liberação de gliotransmissores, moléculas responsáveis por processos de modulação neuronal. O conceito de *sinapse tripartide* continua muito questionado, principalmente porque os avanços neste campo ainda sofrem com algumas limitações metodológicas. De fato, a amplitude e complexidade das funções dos astrócitos são dependentes de sua variedade morfológica, molecular e funcional, bem como também do estado funcional de todo o sistema nervoso central. Ainda que muitas questões cruciais permaneçam sem resposta, o interesse nos estudos sobre os astrócitos vem crescendo em diversos laboratórios de neurociências no mundo. A contribuição relevante da pesquisa básica de cada grupo permite o progresso destes estudos e incentiva o desenvolvimento de novas tecnologias.

6. Perspectivas

São relevantes para a continuidade deste trabalho as seguintes possibilidades:

1. Caracterizar o perfil de mobilização ao Ca^{2+} que promove a secreção de S100B dependente do estímulo com nicotina;

2. Investigar se a nicotina também é capaz de modular a secreção de S100B em um modelo de dano *in vivo*;

3. Verificar o tipo de resposta celular caracterizada pela interação de S100B e seu receptor RAGE após estímulo dos astrócitos com nicotina em condições normais e patológicas;

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